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(54) Title: MONOCLONAL ANTIBODY FRAGMENTS HAVING IMMUNOSUPPRESSANT ACTIVITY

(57) Abstract

The invention comprises monovalent mAb fragments (Fab) of mAb which have the ability of downregulate HLA-DR expression on antigen presenting cells. The Fab fragments can downregulate such HLA-DR expression without the cytotoxicity of the parent mAb or of bivalent fragments ($F(ab)'_2$) of the mAb. The Fab fragments of the invention are therefore potent, class II MHC-specific immunosuppressive compounds without cytotoxic side effects.

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Monoclonal Antibody Fragments having Immunosuppressant Activity

Class II major histocompatibility complex (MHC) molecules bind antigenic peptide fragments, and display them to helper ($CD4^+$) T cells ("Th" cells) (Ref. 1). Monoclonal antibodies (mAb) specific for class II MHC molecules have been shown to be ex-tremely potent selective inhibitors of Th cell responses *in vitro* (Ref. 2). Since their discovery, they have been considered as potential drugs for selective immuno-suppressive treatment of autoimmune disorders, such as rheumatoid arthritis. Initial *in vivo* studies demonstrated the beneficial influence of these mAbs on Th-cell mediated hetero- and autoimmune responses (Refs. 3-6). However, in some cases, the experimental *in vivo* application of class II MHC-specific mAbs was associated with unexpected complications resulting in death of laboratory primates (Refs. 7, 8). The latter observation suppressed the interest in further studies of immunomodulation by MHC-specific mAbs. A recent publication has described that a 10-fold reduction of class II MHC expression in transgenic mice causes Th cell nonresponsiveness due to inefficient antigen presen-tation (Ref.19). This demonstrates that the reduction in the expression of class II MHC correlates with immunosuppression in an *in vivo* model.

In accordance with the invention, it has been surprisingly found that monovalent mAb fragments (Fab) of mAb which have the ability to downregulate HLA-DR expression can them-selves downregulate such HLA-DR expression without the cytotoxicity of the mAb, itself, or of bivalent fragments ($F(ab)'_2$) of the mAb . The Fab fragments of the invention are therefore potent class II MHC-specific immuno-suppressive compounds without cytotoxic side effects.

Before the present invention is described in more specific terms a short description of the figures is given in the following:

Fig. 1 Eff cts of a DR binding competitor peptide and a DR-specific mAb on the EBV transformed B cell line Priess.

Fig. 2 Time course of modulatory and cytotoxic effects of mAb L243 on LG2.

Fig. 3 Duration of modulatory and cytotoxic effects of L243 on LG2 after removal of mAb.

Fig. 4 Downregulation of HLA-DR expression on different APC populations after co-culture with DR specific mAb L243 and its fragments.

Fig. 5 Effects of increasing concentrations of DR specific Fab fragment on the EBV transformed B cell line LG2.

Fig. 6 Effects of prolonged co-culture of L243 and its fragments on LG2 cells.

Fig. 7 Dependency of EBV-LCL cytotoxicity on DR-crosslinking.

Fig. 8 Selectivity of DR downregulation on resting B cells and monocytes/macrophages.

Fig. 9 Selectivity of DR downregulation on B cell blasts.

Fig. 10 Selectivity of DR downregulation on LG2 cells.

Fig. 11 Allotype non-selectivity of DR downregulation by mAb.

Fig. 12 Pan-class II downregulation on TS-10 cells by 1-1C4 Fab.

Fig. 13 Lack of TNFα secretion increase upon co-culture of LG2 and Priess cells with L243 and its fragments.

Fig. 14 Antibody concentration requirement for Th cell inhibition and DR downregulation.

Fig. 15 Effect of anti-DR mAb and Fab fragments on antigen presentation by fixed APC.

Fig. 16 Effect of antigen load on the potency of mAb, Fab, and peptide antagonists.

Fig. 17 Relative effects of Fab and peptide on antigen dose-response curves.

Fig. 18 Effect of class II antagonists on ongoing Th cell response.

Certain monoclonal antibodies (mAbs) which are specific for HLA-DR (Human Leukocyte Antigen of the type "DR"; a class II Major Histocompatibility Complex ("MHC") molecule) can downregulate the expression of HLA-DR molecules on the surface of leukocytes which are antigen presenting cells ("APC") by about 90%. The same mAbs also inhibit the activation of human Th cell clones that require antigen presentation by HLA-DR molecules for activation. The inhibitory potency of such mAbs is several 100 to several 1000 fold higher than that of the currently available peptide antagonists (see Table 1, below). This downregulation of the expression of HLA-DR and the inhibition of the activation of Th cells is a pharmacological activity which results in immunosuppression. This immunosuppression would be useful in the treatment of autoimmune diseases, especially rheumatoid arthritis.

In accordance with the invention, it has been surprisingly found that monovalent, antigen-binding mAb fragments (Fab) of mAbs having the ability to downregulate HLA-DR expression can themselves downregulate such HLA-DR expression without the cytotoxicity of the mAb, itself, or of the bivalent fragments ($F(ab)'_2$) of the mAb . The Fab fragments of the invention are therefore potent, class II MHC-specific immunosuppressive

compounds without cytotoxic side effects. Thus, the present invention comprises a Fab fragment of an anti-HLA-DR mAb wherein said intact mAb is cytotoxic to antigen presenting cells and downregulates HLA-DR expression on the remaining antigen presenting cells. Such a mAb inhibits Th cell activation. The mAbs from which the Fab fragments of the present invention are derived all bind to the first domain of HLA-DR.

Examples of three downregulating mAbs useful in accordance with the invention are:

LB3.1 (mouse IgG_{2b}, pan-DRα1-specific; refs. 9-10);

L243 (mouse IgG_{2a}, pan-DRα1-specific; refs. 10-11; ATCC Accession No. HB55); and

SFR3-DR5 (rat IgG_{2b}, DRB1*110X-specific; ref. 13; ATCC Accession No. HB-151).

In addition, an HLA-DR downregulating mAb, 1-1C4 (mouse IgG_{2a}, ß-chain specific; ref. 14), which additionally downregulates HLA-DQ and -DP, was generated by conventional means as described in Example 22. Thus, the Fab fragments of the above mAbs are encompassed by the present invention.

Consistent with the ability of downregulating mAbs to inhibit Th cell activation, five non-downregulating mAbs, CCCL20 (mouse IgG_{2b}, specific for three DRB1 (ß-chain) allelic forms (DRB1*0101, DRB1*0401, DRB1*0404); ref. 12) and 8D1, 9F1, 9F2, 10F12 (all four mouse IgG1), inhibit Th cell activation only very weakly or not at all.

The active mAbs are cytotoxic to B lymphoblastoid cells and to a small proportion of normal activated B cells. Like the mAbs, the bivalent $F(ab)'_2$ fragments of these mAbs mediate downregulation but are also cytotoxic. However, in accordance with the invention, the monovalent Fab fragments of these mAbs

loose cytotoxicity, but surprisingly retain the downregulating property of the parent mAb.

The anti-HLA-DR mAbs used to obtain the Fab fragments of the invention may be produced by any conventional means, e.g., generally by the procedure first described by Kohler and Milstein. By injection of an antigen into a mouse or rat, rabbit, sheep or the like (preferably a mouse), monoclonal antibodies can be prepared by recovering antibody producing cells from such an immunized animal and immortalizing said cells obtained in conventional fashion like fusion with myeloma cells, e.g., PAI mouse myeloma cells, SP2/0- or SP2/0-Ag14-cells [ATCC No. CRL 1581; ATCC No. CRL 8287][for a general guideline for producing antibodies see, e.g., "Antibodies-A Laboratory Manual" , Harlow & Lee, ed. Cold Spring Harbor Laboratory Press (1988)]. Supernatants of cultures of such hybridomas can then be screened for monoclonal antibodies (mAbs) by conventional procedures like radioimmuno- or enzymimmuno- or dot-immunobinding or immunofluoresence assays. mAbs can be purified from hybridoma supernatants by conventional chromatographic procedures like, for example, ion-exchange chromatography, affinity chromatography on protein A, anti-immunoglobulin-antibodies, or the antigen or a part thereof bound to a solid support, HPLC and the like. The production of a mAb useful in accordance with the invention is shown in Example 22.

For the production of large quantities of mAbs, in accordance with methods well known in the art, hybridomas secreting the desired antibody can be injected intraperitoneally into mice which have been pretreated with, for example, pristane before injection. Up to around 100 mg of a mAb can be produced by the resulting ascites tumors in one mouse. mAbs can be purified from the ascites fluid produced by such tumors by the methods described above.

mAbs can be characterized according to their subclass by known methods, such as by Ouchterlony immunodiffusion. It is also known in the art that mAbs can be modified for various uses,

or fragments thereof can be generated, which are still capable of binding antigen. Such fragments can be generat d, for example, by enzymatic digestion of mAbs with papain, pepsin, or the like.

Immunogen for producing mAb which can be used in accordance with the invention is preferably HLA-DR ß-chain [see e.g. WO92/10589; J. Biol. Chemistry 262, 8748-8758 (1987); sequence information can be obtained also from sequence data bases, for example like Genbank (Intelligenetics, California, USA), European Bioinformatics Institute (Hinxton Hall, Cambridge, GB), NBRF (Georgetown University, Medical Centre, Washington DC, USA) and Vecbase (University of Wisconsin, Biotechnology Centre, Medision, Wisconsin, USA); such sequences can than be used by methods known in the state of the art to produce the antigen for the preparation of the monoclonal antibodies for use in the present invention] which has been purified by conventional means, such as SDS-PAGE.

The identification of the above-described anti-HLA-DR mAbs which are cytotoxic to APC and also downregulate HLA-DR expression may be performed by any conventional means. Preferably, this identification of anti-HLA-DR mAb is performed in accordance with Example 3 where an Epstein-Barr Virus transformed human B-Lymphoblastic Cell Line (EBV-LCL) is used as a model of an APC (particularly, activated B cells). Cultures of at least 1 ml containing about 10^5 EBV-LCL cells per milliliter are preferably used. The culture is incubated with 20 nM of the anti-HLA-DR mAb for 16 hours, and the number of dead cells and the HLA-DR expression by the remaining viable cells is determined by conventional means. For the purposes of this invention, cytotoxicity and downregulation are defined as follows: 1) the mAb is cytotoxic to antigen presenting cells if, under the above conditions, at least 25%, preferably 40%, of the EBV-LCL antigen presenting cells are killed by the intact mAb; and 2) the mAb downregulates HLA-DR expression on antigen presenting cells if, under the above conditions, it reduces the number of HLA-DR molecul s on the surface of the EBV-LCL antigen presenting c lls which remain alive by at least an average of 50%, .

The EBV-LCL antigen presenting cells are labeled to detect the dead cells and to measure the HLA-DR expression of the remaining viable cells by conventional immunofluoresence. The cells are analyzed using a flow cytometer (e.g., FACScan, Becton-Dickinson, San Jose, California), and the percent dead cells and reduction of HLA-DR expression on the remaining cells is calculated by conventional means, preferably using the software normally used with the flow cytometer (e.g., LYSIS II software with the FACScan cytometer).

The EBV-LCL used to screen for monoclonal antibodies having the desired properties are not critical. Any conventional EBV-LCL may be used in accordance with the invention. Examples of EBV-LCL useful in accordance with the invention are Priess (ECACC (Salisbury, UK) Accession No. 86052111), LG2 (*Istituto Nazionale Per La Ricerca Sul Cancro* (Genova, Italy) Accession No. G201 12301), and TS-10 (ECACC (Salisbury, UK) Accession No. 85102911).

The Fab fragment of the above-described mAbs may be produced by any conventional means. The Fab fragment may be produced by digestion of the parent mAb by pepsin and isolating the Fab fragments by means known in the art (e.g., Andrew and Titus, "Fragmentation of immunoglobulin G", *Current Protocols in Immunology*, Coligan et al., eds. (Greene & Wiley 1994)). Accordingly such a process and a Fab fragment whenever prepared by such a process are also an object of the present invention.

Preferably, the Fab fragments of the invention are produced by a recombinant cell line which expresses a gene which encodes the desired Fab fragment. Such recombinant cell lines may be produced by any conventional means. For example, the portion of the gene encoding the Fab fragment would be cloned by conventional means from the hybridoma which secretes the parent mAb of the Fab fragment. The Fab fragment cDNA may then be incorporated by conventional means into an expression vector, which in turn, is used to transfect an appropriate c ll line.

The preferred Fab fragments of the invention are "humanized" so that they are less antigenic when administered to humans than a Fab fragment that is produced directly from an animal, preferably murine, mAb. The method by which humanized Fab fragments of the invention are produced is not critical. Any conventional means known in the art may be used. Such methods utilize the fact that any immunoglobulin (Ig), such as a monoclonal antibody, consists of a constant domain and of a variable domain where the antigen binding occurs. The variable domain, in turn, consists of six complementarity-determining regions ("CDR's") embedded in a framework region (three CDR's on each of the light chain and the heavy chain of the Ig). (Ref. 42). It is the CDR's which are responsible for the specificity of the mAb. Since mAbs are of murine or other animal species origin, humanization of mAbs is performed essentially by replacing at least one, but preferably all six, of the CDR's of a human immunoglobulin with the corresponding CDR's of an animal mAb which has the desired specificity. Thus, the human Ig serves as the framework for the animal CDR's. In such procedures, the animal mAb, usually murine, is described as the "donor" and the human Ig is described as the "acceptor."

Further "fine tuning" of the amino acid sequence of the humanized mAb as described in the art may also be performed to optimize antigen specificity of the humanized mAb. For example, International Patent Application Publication No. WO 90/7861 discloses that a panel of 10-20 human Ig's should be screened, and the human Ig whose variable region has the greatest degree of homology with the variable region of the murine donor mAb, typically 65-70% or higher homology, should be used as the acceptor. Further substitutions of donor amino acids for acceptor amino acids (typically about three substitutions) may be made outside the CDR's based upon the four criteria disclosed in WO 90/7861 at pages 12-15.

In another example of humanization known in the art, EP 0 620 276 discloses a hierarchy of particular substitutions which may be made to th acceptor Ig outside the engrafted donor CDR's

in order to increase the specificity of the humanized mAb. Such substitutions are disclosed as obviating the need to select a human acceptor Ig whose variable region has a high degree of homology with the variable region of the donor mAb. A specific protocol for humanization is provided in EP 0 620 276 at pages 8-9.

Methods for generating DNA sequences for the expression in a host cell of a humanized intact mAb useful for obtaining the Fab fragments of the invention, or for the expression of a humanized Fab fragment of the invention, are known in the art and are not critical. Such methods include, e.g., site-directed mutagenesis, constructing the whole variable region using overlapping oligonucleotides which incorporate the animal CDR's on a human framework, and using PCR grafting. (WO 90/7861, EP 0 620 276, Ref. 43).

Thus, the invention may be further described as a Fab fragment comprising a immunoglobulin Fab fragment and six complementarity-determining regions which are contained within said immunoglobulin Fab fragment, wherein from one to six of said complementarity-determining regions are the complementarity-determining regions of a monoclonal antibody having the following properties:

1) the monoclonal antibody binds to the first domain of HLA-DR,

2) the monoclonal antibody is cytotoxic to antigen presenting cells which express HLA-DR,

3) the monoclonal antibody downregulates HLA-DR expression on the antigen presenting cells.

The preferred embodiment of the invention is a humanized Fab fragment wherein, in accordance with the above, the immunoglobulin Fab fragment is human and the monoclonal antibody having prop rties 1-3 is animal, preferably murine. This humanized Fab fragm nt would be a human immunoglobulin Fab

fragment in which from on to six of the CDR's contained therein had be n replaced by the corresponding CDR's of the animal mAb. Thus, the preferred Fab fragment of the invention is a Fab fragment comprising a human immunoglobulin Fab fragment and six complementarity-determining regions which are contained within said human immunoglobulin Fab fragment, wherein from one to six of said complementarity-determining regions are the complementarity-determining regions of a monoclonal antibody having properties 1-3, above.

It is preferred that this humanized Fab fragment of the invention contain all six of the animal mAb CDR's.

In the case where the immunoglobulin Fab fragment is of animal origin, it is contemplated that the Fab fragment of the invention can be a Fab fragment of the monoclonal antibody, itself, which has properties 1-3, above. Such a Fab fragment would be useful as an intermediate for use in obtaining the animal CDR's that will be contained in the preferred humanized Fab fragment of the invention.

The preferred Fab fragments of the invention have properties similar to Fab fragments obtained from monoclonal antibodies LB3.1, L243, SFR3-DR5 and 1-1C4, described above. Since the Fab fragments of the invention inhibit Th cell activation, they may be used in the treatment of various diseases in which activated Th cells are a source of disease damage or symptoms. One such disease is rheumatoid arthritis. (Ref. 33). Downregulating HLA-DR on APC of a patient with rheumatoid arthritis, and thereby inhibiting Th cell activation in such a patient, would slow or stop the progression of the disease. Inhibiting Th cell activation in a patient with rheumatoid arthritis would also relieve symptoms, such as pain and inflammation, by decreasing or halting the release of mediators which are the cause of those symptoms.

Thus, the invention also comprises Fab fragment as described herein and their use as a therapeutically active agent, especially as an immunsuppressive agent and more specifically for the treatment of rheumatoid arthritis and a method of suppressing the

immune response of a patient comprising administering a therapeutically effective amount of a Fab fragment of the invention to a patient in need of such treatment. Th invention further comprises a method of treating rheumatoid arthritis in a patient by administering a therapeutically effective amount of a Fab fragment of the invention to a patient in need of such treatment. The amount of Fab fragment to be administered may be determined by any conventional means. Also, the administration of the Fab fragment of the invention may be performed by any conventional means.

Administration of a Fab fragment of the invention is preferably accomplished using a pharmaceutical composition of the invention (described below). Administration is preferably performed parenterally (subcutaneously, intramuscularly, or intravenously), especially intravenously. The dosage required to inhibit Th cell activation in a patient, and thereby treat rheumatoid arthritis, may be determined by any conventional means, e.g., by dose-limiting clinical trials. However, a dosage of about 1-10 mg/day i.v., especially about 3-7 mg/day, particularly about 5 mg/day, is preferred (Refs. 34, 35), preferably delivered as a bolus. Treatment is preferably once daily for one week or less, however daily treatment may be continued for up to three weeks, if necessary.

The Fab fragments of the invention may be formulated as a fluid pharmaceutical composition, e.g. for parenteral administration comprising the Fab fragment of the invention dissolved in a conventional pharmaceutically acceptable fluid carrier material. The composition may further comprise other pharmacologically active substances. Preferably, the composition contains about 0.5-5 mg/ml of a Fab fragment of the invention, especially about 1-2 mg/ml. The preferred fluid carrier is sterile, physiological saline.

The results described below in Examples 1-7 indicate that inhibition of Th cell activation by HLA-DR-specific mAb can operate by multiple actions: (a) elimination of available APC by direct cytotoxicity, (b) reduction of available HLA-DR molecules on

the remaining viable APC by downregulation of cell surface expression, (c) hindranc of class II MHC-TcR interaction. The Th cell inhibition may also occur by mAb blocking the peptide binding groove on DR molecule, rendering it inaccessible for antigen (30). It is therefore understandable why mAbs are superior Th cell inhibitors to the current peptide antagonists, the effect of the latter being exclusively based on blocking the antigen binding site of HLA-DR molecules.

The mechanisms underlying cytotoxicity and class II downregulation by antibody remain to be investigated. However, it is clear that cytotoxicity requires crosslinking, whereas downregulation is also achieved with monovalent Fab fragments of the invention without the cytotoxicity of the parent mAb. This difference permits the separation of these two properties by antibody fragmentation. Assuming that the previously observed side effects of anti-class II Ab (Refs. 7, 8) were, at least in part, associated with direct cytotoxicity, the Fab fragments of the invention provide for the therapeutic use of these antibodies as immunosuppressants, without adverse effects.

Examples

Example 1

Isotype specificity of anti-DR mAb.

The precise specificities of mAbs were determined by their ability to stain mouse cell lines transfected with human HLA class II genes.

Methods: Mouse fibroblast lines transfected with indicated human class II genes (15), as well as untransfected host cells (Lmtk$^-$) were stained by the standard indirect immunofluoresence using DR-specific mAb and FITC-conjugated goat-anti mouse Ig (Southern, Birmingham, Alabama) as the primary and secondary reagents, respectively. Samples were analyzed on a FACScan flow cytom ter (Becton-Dickinson, San Jose, California). R sults are shown in **Table**

1. "+" and "-", presence and absence of antibody binding, respectively; "NT", not tested.

Conclusion: Antibodies 8D1, 9F1, 9F2 and 10F12 are pan-DR specific, whereas 1-1C4 can recognize all three human class II isotypes (DR, DP and DQ).

Table 1. Staining pattern of pan-DR specific mAb

Mouse fibroblast line	HLA class II expressed	mAb				
		1-1C4	8D1	9F1	9F2	10F12
Lmtk-	-	-	-	-	-	-
L57.23	DRA*0101/ DRB1*0101	+	NT	+	+	+
L243.6	DRA*0101/ DRB1*0401	+	+	+	+	+
L164.11	DRA*0101/ DRB1*0402	+	NT	NT	NT	NT
L259.1	DRA*0101/ DRB1*0403	+	NT	NT	NT	NT
L300.7	DRA*0101/ DRB1*0404	+	NT	+	NT	NT
L241.2	DRA*0101/ DRB1*0701	+	NT	+	NT	NT
L91.7	DRA*0101/ DRB1*1101	+	NT	+	+	+
L167.2	DRA*0101/ DRB1*1401	+	NT	NT	NT	NT
L182.1	DRA*0101/ DRB1*1402	+	NT	NT	NT	NT
L105.1	DRA*0101/ DRB3*0202	+	NT	NT	NT	+
L168.2	DRA*0101/ DRB3*0101	+	NT	NT	NT	NT
L257.6	DRA*0101/ DRB4*0101	+	NT	NT	NT	NT

L256.12	DPA1*0202X/ DPB1*0201	+	NT	NT	NT	NT
L25.4	DPA1*0103/ DPB1*040X	+	-	-	-	-
L21.3	DQA1*0201/ DQB1*0602	+	NT	NT	NT	NT

Example 2

Chain and domain specificity of anti-HLA-DR mAbs.

Methods: Using the standard gene cloning, recombination and transfection technology (10), two mouse B cell lines expressing chimeric human/mouse class II molecules were generated. Transfectant M12.C3.25 was derived from a mouse class II-negative host line M12.C3 (15), and expresses MHC product composed of α1 and ß1 domains derived from a human HLA-DRA*0101/DRB1*0401 molecule, and α2 and ß2 domains of a mouse I-E^d protein, to which DR-specific reagents do not bind. Transfectant CH27.105 was derived from a mouse class II-positive host line CH27 (16), and expresses the original mouse $E\beta^k$ chain associated with the chimeric human/mouse α chain described above. Standard indirect immunofluoresence staining was performed using indicated IgG_2 and IgG1 antibodies, in conjunction with protein A-FITC (Boehringer, Mannheim, Germany) and goat anti-mouse IgG_1-FITC (Southern, Birmingham, Alabama), respectively. Samples were analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, California). Results are shown in **Table 2** (presentation is as in Table 1).

Conclusion: The results confirm the previously reported DRα1 specificity of LB3.1 and L243 (10). Antibodies 8D1 and 1-1C4 are ß1-specific, whereas the epitope recognized by CCCL20 appears to be ß2 domain-dependent. Remaining reagents (9F1, 9F2, 10F12) seem to bind determinant(s) expressed by the second HLA-DR domain(s). Corresponding epitope of anti-DRB1*110X mAb could not be mapped using these transfectants, since SFR3-DR5 does not recognize DRB1*0401 allotype.

Table 2. Binding of anti-DR mAb to mouse B cell lines transfected with chimeric human-mouse recombinant class II genes.

Mouse cell line:	M12.C3	M12.C3.25	CH27	CH27.105
Expression of **human** classs II-derived protein:	-	**DRα1-Eα2/DRß1-Eß2**	-	**DRα1-Eα2/Eß1-Eß2**
mAb: LB3.1	-	+	-	+
L243	-	+	-	+
1-1C4	-	+	-	-
8D1	-	+	-	-
9F1	-	-	-	-
9F2	-	-	-	-
10F12	-	-	-	-
CCCL20	-	-	NT	NT

Effect of anti-DR mAbs and their fragments on HLA-DR expr ssion and cell viability

The DR expression and viability of antigen presenting cells (APC) pre-cultured with either a DR-binding competitor peptide aXA (17), or DR-specific mAb was examined at respective concentrations that, in the case of aXA and two mAb (LB3.1, 1-1C4), blocked antigen presentation to Th cells. As APC, an Epstein-Barr virus transformed human B-lymphoblastic cell line (EBV-LCL) was used .

Example 3

Effects of a DR binding competitor peptide and a DR-specific mAb on an EBV-LCL

Methods: EBV-LCL (Priess, ECACC (Salisbury, UK) Accession No. 86052111) (10^5 cells/ml) were cultured in the presence of DR-specific mAb (LB3.1, 1-1C4, CCCL20) or peptide aXA (aXAAAKTAAAAa-NH_2; ref. 17) at the concentrations indicated in **Fig. 1** for 16 hours. Cells were subsequently washed and stained by the standard indirect immunofluoresence using DR-specific mAb [LB3.1 (*a*), CCCL20 (*b*), 1-1C4 (*c*)], and FITC-conjugated goat-anti mouse Ig (Southern Biotechnology Associates Inc., Birmingham, Alabama) as the primary and secondary reagents, respectively. Samples were analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, California). The results are shown in **Fig. 1**. The X-axis represents HLA-DR expression, and the Y-axis fluorescence of propidium iodide-stained dead cells, both in arbitrary fluorescence units. "Background" represents control cell population cultured for 16 hours in normal medium and subsequently labeled without primary reagent, respectively.

Conclusion: Co-culture with peptide aXA (Fig. 1*a*) did not significantly affect either cell viability or DR expression, whereas DR specific mAb LB3.1 (Fig. 1*a*) and 1-1C4 (Fig. 1*c*) induced high cytotoxicity, as well as reduced DR expression on the remaining viable cells. However, these properties were not shar d by all

anti-DR mAbs, since CCCL20 affected neith r viability nor DR xpression, ven at increased concentration (Fig. 1*b*).

DR downmodulation and cytotoxicity on EBV-LCL could be induced with two more DR specific mAb, L243 and SFR-DR5, whereas four other anti-DR mAb (8D1, 9F1, 9F2 and 10F12) were neither cytotoxic, nor reduced DR expression (data not shown).

Example 4

Time course of modulatory and cytotoxic effects of mAb L243 on LG2.

Methods: EBV-LCL LG2 (*Istituto Nazionale Per La Ricerca Sul Cancro* (Genova, Italy) Accession No. G201 12301) (10^5 cells/ml) were cultured in the presence of DR-specific mAb L243 (Becton-Dickinson) at the concentration of 10 nM for the indicated period of time. Cells were subsequently washed and stained by the standard indirect immunofluoresence using DR-specific mAb L243 and FITC-conjugated goat-anti mouse Ig (Southern, Birmingham, Alabama) as the primary and secondary reagent, respectively. Dead cells were stained with propidium iodide, and samples were analyzed on a FACScan flow cytometer. The results are shown in **Fig. 2.** Histograms represent relative number of dead cells (light) and live cells expressing decreased amounts of HLA-DR (dark).

Conclusion: Kinetic *in vitro* studies demonstrated detectable cytotoxicity of mAb after 2 hours, and plateau toxicity after 8 hours. Downregulation of DR became detectable after 4 hours, reaching its peak after 8 hours.

Example 5

Duration of modulatory and cytotoxic effects of L243 on LG2 after removal of mAb.

Meth ds: EBV-LCL LG2 (10^5 cells/ml) were cultur d in the presence of DR-specific mAb L243 (Becton-Dickinson) at the

concentration of 10 nM for the indicated period of time. Antibody was removed by 3x washing and cell culture was resumed in the sam volume of the fresh medium, as indicated. Cells were subsequently washed and stained by the standard indirect immunofluoresence using DR-specific mAb L243 and FITC-conjugated goat-anti mouse Ig (Southern, Birmingham, Alabama) as the primary and secondary reagent, respectively. Dead cells were stained with propidium iodide, and samples were analyzed on a FACScan flow cytometer. The results are shown in **Fig. 3.** Histograms represent relative number of dead cells (light) and live cells expressing decreased amounts of HLA-DR (dark).

Conclusion: Antibody induced DR downregulation lasts for 16 hours after removal of mAb.

Example 6

Downregulation of HLA-DR expression on different APC populations after co-culture with DR specific mAb L243 and its fragments.

The effects of anti-DR mAb on different, untransformed MHC class II positive cell subpopulations: resting and activated B cells, monocytes/macrophages and activated Th cells was further examined .

Methods: Isolation of B cells and monocytes/macrophages from fresh peripheral blood was done by removing T cells plus monocytes or B cells (where applicable) prior to culture, using magnetic beads (Dynal) precoated with mAb specific for CD3 (SK7) and CD14 (MΦP9) or CD19 (4G7), respectively (Becton-Dickinson). Th and B cell blasts were generated by 3-5 days *in vitro* stimulation of peripheral blood mononuclear cells with phytohemagglutinin (1 µg/ml; Sigma) and pokeweed mitogen (2.5 µg/ml; Sigma), respectively. L243 was digested by pepsin and papain in order to isolate $F(ab)'_2$ and Fab fragments, respectively as per ref. 18. Undigested antibodies and their Fc fragments were removed by affinity chromatography on protein G columns. APC (10^5 cells/ml) were cultured in the presence of equivalent

concentrations of complete L243 antibody (10 nM) or its F(ab)'$_2$ (10 nM) and Fab (20 nM) fragments for 16 hours, as indicated in **Fig. 4**. Cells were subsequently washed and stained as in Example 3. Results are shown in **Fig. 4**. The X-axis represents HLA-DR expression, and the Y-axis fluorescence of propidium iodide-stained dead cells, both in arbitrary fluorescence units. Numbers indicate percentages of cells in the corresponding quadrants. "DR-FITC staining" and "Background FITC staining" represent control cell populations cultured for 16 hours in normal medium and subsequently labeled with and without primary reagent, respectively.

Conclusion: The cell surface expression of DR molecules decreased in all populations in the presence of a complete mAb, as well as its bi- and monovalent fragments F(ab)'$_2$ and Fab, respectively. The extent of DR reduction was high in B cells (80-90%), intermediate in monocytic APC (50-65%), and low (less than 50%) in Th cell blasts. Co-culture of EBV-LCL and preactivated B cell blasts with bivalent anti-DR reagents [complete mAb and F(ab)'$_2$] resulted in high (60-70%) and marginal (5-15%) cytotoxicity, respectively, whereas monovalent fragments (Fab) only induced downregulation without significant cell death. The cytotoxic effect was completely absent in subpopulations of resting B cells, activated Th lymphocytes and monocytes/macrophages preincubated with any form of DR-specific mAb used in these experiments.

Similar profiles (partly shown in further sections) were obtained using complete mAb 1-1C4 and LB3.1 and their Fab fragments. [F(ab)'$_2$ fragment of LB3.1 antibody cannot be generated because its isotype does not permit papain digestion].

Example 7

<u>Effects of increasing concentrations of DR specific Fab fragment on the EBV transformed B cell line LG2.</u>

The difference in terms of cytotoxicity between mono- and bivalent mAb reagents was explored to determine if it was only

quantitative, or if it was due to a qualitatively different mechanism of action.

Methods: As in Examples 3 and 6. Results are shown in **Fig. 5.** Presentation is as in Fig. 1.

Conclusion: Increased concentration of L243-Fab failed to induce any significant decrease in APC viability.

Example 8

Effects of prolonged co-culture of L243 and its fragments on LG2 cells.

Methods: APC (10^5/ml) were cultured in the presence of equivalent concentrations of complete L243 antibody (10 nM) or its Fab fragment (20 nM) for the indicated period of time. Cells were subsequently washed and stained as in Example 6. Results are shown in **Fig. 6**. Presentation is as in Fig. 2.

Conclusion: Prolonged co-culture period failed to induce any significant decrease in APC viability.

Example 9

Cytotoxicity of EBV-LCL depends on DR-crosslinking.

Methods: EBV-LCL (Priess) (10^5 cells/ml) were cultured for 16 hours in the presence of L243 Fab (20 nM) fragments and goat anti-mouse IgG precoated magnetic beads ("anti-Ig"; Dynal), in order to crosslink cell membrane bound Fab fragments, as indicated. Cells were subsequently washed and stained as in Example 6. Results are shown in **Fig. 7**. Presentation is as in Fig. 1.

Conclusion: Crosslinking of HLA-DR-bound Fab generates cytotoxic effect on EBV-LCL.

From the results described thus far, it was concluded that:

(a) HLA-DR expressed on any mononuclear blood cell type can be downregulated upon its ligation by specific mAb;

(b) unexpectedly, DR ligation by monovalent reagents (Fab) can also lead to DR downregulation;

(c) since cytotoxicity is apparent only on EBV, a model of activated B cells, and mitogen preactivated B cells, it is probably dependent upon the activated stage of B cells; and

(d) cytotoxicity is the consequence of DR-crosslinking by bivalent ligands [complete mAb and $F(ab)'_2$, crosslinked Fab], and does not require the Fc portion of antibody.

Previous studies have shown that ligation of class II molecules with whole mAb or T-cell receptors signals a series of changes in APC such as cytokine secretion (refs. 19-20), modulation of cell growth and immunoglobulin secretion (refs. 21-28), upregulation of costimulatory (ref. 15) and cell adhesion molecules (refs. 20, 29), and downregulation of CD23 (ref. 25). It was therefore important to establish whether the decrease of DR expression upon pre-culture with mAb was restricted to the class II molecule ligated by the antibody, or it was part of a globally induced downregulation of numerous cell surface proteins. The expression of HLA class II isotypes DP and DQ, HLA class I molecules, and an MHC unrelated adhesion protein CD18, was analyzed on different APC subpopulations after co-culture with $F(ab)'_2$ or Fab fragments of L243 .

Example 10

<u>Selectivity of DR downregulation on resting B cells and monocytes/macrophages.</u>

Methods: As in Example 6. Standard indirect immunofluoresence staining by mAb specific for HLA-DP (clone B7/21), -DQ, (SK10), CD18 (L130) (all of IgG_1 subclass, Becton-Dickinson) and anti-HLA

class I (W6-32 IgG2a, Accurate, Westbury, New York) reag nts was performed in conjunction with goat anti-mouse-IgG_1-FITC and FITC-labeled protein A (Boehringer), respectively. This procedure excludes additional indirect FITC-labeling of residual membrane-bound DR-specific antibody fragment. Results are shown in **Fig. 8**. Presentation is as in Fig. 4. "Control" represents cell population cultured for 16 hours in normal medium and subsequently labeled with Protein A and FITC-conjugated goat anti-mouse IgG1, together.

Conclusion: While DR molecules exhibited reduced expression, DP, DQ, class I, and CD18 molecules remained unaffected in both resting B cells and monocytes/macrophages precultured with $F(ab)'_2$ fragments.

Example 11

Selectivity of DR downregulation on B cell blasts.

Methods: As in Example 10. Results are shown in **Fig. 9**. Presentation is as in Fig. 4.

Conclusion: While DR molecules were downregulated, the expression of DP, DQ, class I, and CD18 molecules remained unaffected in B cell blasts precultured with $F(ab)'_2$ fragments.

Example 12

Selectivity of DR downregulation on LG2 cells.

Methods: As in Example 10. Results are shown in **Fig. 10**. Presentation is as in Fig. 4.

Conclusion: While the expression of DR molecules appeared to be selectively reduced upon ligation by Fab, bivalent $F(ab)'_2$ fragments induced a significant downregulation of other class II isotypes, class I molecules and CD18. Since the level of non-selective modulation is only two-fold and DR reduction is ten-fold,

it is reasonable to assume that the decrease in expression of other cell surface molecules is associated with a general noxiousness of bivalent reagents on EBV-LCL.

Example 13

Allotype non-selectivity of DR downregulation by mAb.

Modulatory mAb LB3.1, L243 and 1-1C4 are pan-DR specific, *i.e.* they do not distinguish between different allelic forms of HLA-DR. Therefore, they are not suitable for testing allotype specificity of DR downregulation. However, this question could be addressed using downregulatory mAb SFR3-DR5, which is exclusively specific for DRB1*110X (formerly DR5; ref. 13).

Method: B cells (isolated from fresh peripheral blood of a heterozygous *DRB1*0101/1101X* donor as in Example 6) were cultured in the presence of DRB1*110X-specific mAb SFR3-DR5 (20% hybridoma supernatant fluid; ref 14). Cells were subsequently washed and stained by standard indirect immunofluoresence with either DRB1*110X-specific mAb SFR3-DR5, or DRB1*1101-specific mAb CCCL20 (12), in conjunction with goat anti-mouse & rat Ig (Southern), as indicated. Results are shown in **Fig. 11**. Presentation is as in Fig. 4.

Conclusion: DRB1*110X-specific reagent SFR3-DR5 induced downregulation of both DR allomorphs expressed by heterozygous resting B cells, affecting DR allotype not recognized by the mAb, too. Thus, the DR downregulation does not appear to be allotype specific.

Example 14

Pan-class II downregulation on TS-10 cells by 1-1C4 Fab.

Since mAb 1-1C4 can recognize ß chain of all three HLA class II isotypes (DR, DP, DQ) it was important to test whether it is capable of reducing their expression accordingly.

Meth ds: EBV-LCL TS-10 (ECACC (Salisbury, UK) Accession No. 85102911) were cultured in the presence of 1-1C4 Fab (20 nM), anti-DP mAb (10 nM) or anti-DQ mAb (10 nM) for 16 hours, as indicated. Cells were subsequently washed and stained as in Examples 6 and 10. Results are shown in **Fig. 12**. Histogram staining profiles of live gated cells are shown. The X-axis represents fluorescence intensity in arbitrary units, and the Y-axis relative cell number. Open histograms represent control cell populations labeled with the respective secondary reagents.

Conclusion: 1-1C4 Fab downregulated expression of all three class II isotypes (DR, DP, DQ) with the reduction intensity comparable to the one achieved by isotype-specific mAb (anti-DP, anti-DQ). This pan-class II downmodulation by 1-1C4 Fab was selective, since HLA class I and CD18 molecules remained unaffected. Therefore, in addition to being useful for treating HLR-DR-linked indications such as rheumatoid arthritis, Fab fragments of the invention obtained from pan-class II mAbs such as 1-1C4 would be useful for treating diseases linked to HLA-DP and -DQ expression, such as multiple sclerosis, type I diabetes mellitus, myasthenia gravis, erythematosus, organ transplant rejection, and graft versus host disease. (Refs. 36-41)

Example 15

Lack of TNFα secretion increase upon co-culture of LG2 and Priess cells with L243 and its fragments.

Previous studies have shown that crosslinking of DR molecules by L243 on EBV-transformed B cell line JY increased the secretion of TNFα (20). Since this could be a possible mechanism for cytotoxicity, the TNFα release by Priess and LG2 cells co-cultured with the same mAb L243 and its fragments was measured.

Meth ds: APC (10^5 cells/ml) were cultured in the presence of equivalent concentrations of compl te L243 antibody (10 nM) or

its F(ab)'$_2$ (10 nM) and Fab (20 nM) fragments for 16 hours, as indicated. TNFα concentration in culture sup rnatants was measured using internally controlled standard ELISA kit (T cell Diagnostics, Cambridge, Massachusetts). Cytotoxic and DR-modulatory effects of monoclonal reagents were confirmed by immunofluoresence as in Example 4 (data not shown). Results are shown in **Fig. 13.**

Conclusion: No significant TNFα increase was detected in these cultures. Therefore, the mechanism of cytotoxicity, as well as that of selective DR downmodulation remain to be investigated.

Inhibition of Th cell response with anti-DR mAbs and their fragments

Example 16

Inhibition of Th cell response with anti-DR mAb, their fragments and DR-binding peptides.

Methods: CD4 positive Th cell clones NBHAC25, KMHA25 and DSHABB10 prepared as per Ref. 32 respond to influenza virus hemagglutinin (HA) peptide HA307-319 (PKYVKQNTLKLAT) presented by DRA/DRB1*0101, DRA/DRB1*0401 and DRA/DRB1*0401, respectively. Th cells were incubated with mitomycin C-treated APC, antigen (134 nM), and inhibitors. Antigen specific Th cell proliferation was measured by the standard ^{3}H-thymidine incorporation assay. Results are shown in **Table 3.**

Conclusion: The mAbs LB 3.1, L243 and 1-1C4, *i.e.*, those inducing downregulation of DR molecules, were also potent inhibitors of Th helper cell activation. Conversely, mAb that did not downregulate DR (CCCL20, 8D1, 9F1, 9F2, 10F12), either failed to affect Th cell responses, or induced marginal inhibitory effect (Table 3). Thus, the capability of DR downregulation correlates with the inhibitory activity. In addition, these results indicate that downregulatory

mAb tend to recognize epitopes located on peptide-binding (α1 and ß1) domains of class II molecule.

Table 3. Inhibition of T cell response with anti-DR mAb, their fragments, and DR-binding peptides.

Th clone	APC: DRB1*[a]	Antigen	Inhibitor	Inhibitor's biological activity				
				DR domain specificity	DR down-regulation	Max. % inhibit.[b]	IC_{50}[c] (or range; nM)	IC_{94}[d] (or range; nM)
NBIIAC25	LG2:0101	IIA307-319	LB3.1 mAb	α1	+	100	3-8	6-60
			LB3.1 Fab	α1	+	100	2-8	20-120
	PBMC:0101	IIA307-319	LB3.1 mAb	α1	+	100	0.9	30
			LB3.1 Fab	α1	+	100	70	500
		Native IIA	LB3.1 mAb	α1	+	100	<0.1	0.6
			LB3.1 Fab	α1	+	100	0.8	60
	LG2:0101	IIA307-319	L243 mAb	α1	+	100	3-8	6-20
			L243 F(ab)'$_2$	α1	+	100	4	30
			L243 Fab	α1	+	100	7-40	40-180
			1-1C4 mAb	ß1	+	100	1-4	6-20
			1-1C4 Fab	ß1	+	100	4-12	20-60
			CCCL20 mAb	ß2	-	0-26	N/A[e]	N/A
			8D1 mAb	ß1	-	0-18	N/A	N/A
			9F1 mAb	2	-	31-38	N/A	N/A
			9F2 mAb	2	-	42-47	N/A	N/A
			10F12 mAb	2	-	42-52	40	N/A
			aXA/aXRA peptide[f]	α1ß1	-	48-78	240-270,000[g]	N/A

			m/1-8 peptide[h]	α1ß1	-	74	2,100	N/A
KMHA25	Priess: 0401	HA307-319	LB3.1 mAb	α1	+	100	1.8-3	6-20
	PBMC: 0401	Native HA	LB3.1 mAb	α1	+	100	0.1	0.6
DSHABB10	Priess: 0401	HA307-319	LB3.1 mAb	α1	+	100	4	18
			1-1C4 mAb	ß1	+	100	3.8	20
			1-1C4 Fab	ß1	+	100	17	80
			L243 Fab	α1	+	100	18	180

[a] DR molecules that present antigen to Th clones.

[b] Achieved at 60 nM for mAb and their fragments, and 10 μM for antagonist peptides.

[c] Concentration required for 50% inhibition of antigen specific proliferative Th response. Upper and lower value from 3 to 4 experiments.

[d] Concentration required for 94% inhibition of antigen specific proliferative Th response. Upper and lower value from 3 to 4 experiments.

[e] Not applicable: IC_{50} and IC_{94} values could not be established since the measured inhibition levels were below 50% and 94%, respectively.)

[f] aXA (peptide CY760.50, aXAAAKTAAAAa-NH_2; ref. 17) and aXRA (aXRAMKTLAAAa-NH_2) equivalent for T cell inhibitory capacity.

[g] Depending on the antigen load (HA307-319 from 13.4 pM to 134 nM).

[h] Ac-YRAMATLA-NH_2.

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Example 17

Antibody concentration requirement for Th cell inhibition and DR downregulation.

To further explore the correlation demonstrated in Example 16, the antibody concentration requirement for downregulation and inhibition was determined.

Methods: Th cell clones KMHA25 and DSHABB10 (see in Table 3) were incubated with mitomycin C-treated Priess cells as APC, HA307-319 peptide as antigen (330 nM), and the indicated concentrations of mAb LB3.1. Antigen specific Th cell proliferation (upper panel) was measured by ^{3}H-thymidine incorporation 3 days later. Flow cytometry of Priess cells with mAb LB3.1 (lower panel) was performed as in Example 3. Results are shown in **Fig. 14**.

Conclusion: A near complete inhibition of Th responses was achieved with mAb concentration which induced 90% downregulation of DR in about 2/3 of APC (without killing them), suggesting a similar concentration requirement for both phenomena.

Example 18

Effect of anti-DR mAb and Fab fragments on antigen presentation by fixed APC.

It was investigated whether DR downregulation is the only mechanism involved in inhibition of Th cell response.

Methods: APC (LG-2) were fixed with glutaraldehyde (Fluka Chemie, Buchs, Switzerland). Response of Th cell clone NBHAC25 to mitomycin treated ("My") or fixed ("Fix") LG-2 plus HA307-319 (134 nM) in the presence of mAb or Fab was measured as in Example 17. Results are shown in **Fig. 15**.

Conclusion: Anti-class II mAbs and their Fab fragments can also inhibit Th cell response to peptide antigen presented on fixed (*i.e.*, dead) APC, where downregulation of class II molecules is not possible. Thus, at least one additional mechanism, most likely steric hindrance of class II- Th cell antigen receptor (TCR) interaction, plays a role in Th cell inhibition.

Example 19

Effect of antigen load on the potency of mAb, Fab, and peptide antagonists.

The relative efficiency of anti-class II mAbs, their fragments, and peptide binding site antagonists in inhibition of Th cell response was compared.

Methods: As in Example 15. Th clone NBHAC25; APC LG-2. Upper panel HA 307-319 13.4 nM, lower panel 330 nM. Peptides as in Table 3. Results are shown in **Fig. 16**.

Conclusion: Antibodies and Fab fragments are comparable in inhibitory capacity, the latter being only marginally less efficient than the former. Both were, however, several hundred fold more efficient than peptides. It is important to note that increasing the antigen concentration (30 fold higher in the lower than in the upper panel of Fig. 16) rendered the peptide antagonist less efficient, but did not affect the potency of mAb. This observation is explained by differences in the mechanism of inhibition: whereas peptides directly compete with the antigen for class II binding sites, antibodies downregulate class II expression as well as hinder MHC-TCR interaction.

Example 20

Relative effects of Fab and peptide on antigen dose-r sponse curves.

The ability of Fab fragments and peptides to inhibit Th cell response to a wide range of antigen concentrations was compared.

Methods: As in Example 17. Th clone, NBHAC25; APC, LG-2; HA307-319 from 13.4 pM to 134 nM; Fab, 100 nM; peptide aXA, 100 µM. Results are shown in **Fig. 17**.

Conclusion: It is clear from the data that Fab fragments have the ability to inhibit Th cell response at ≈1000 fold higher antigen concentrations than peptides, and this difference remains constant over the whole range of antigen concentrations.

Example 21

Effect of class II antagonists on ongoing Th cell response.

It was important to establish whether different kinds of class II antagonists can interfere with an ongoing Th cell response. Although this question cannot be properly investigated within the short time frame of *in vitro* Th cell response, it was attempted to make a comparison between Fab fragments and peptides added at different time points to the APC-antigen-Th cell system.

Methods: As in Example 17. Th clone, NBHAC25; APC, LG-2; HA307-319, 4 nM. Incubation of APC with HA from -2 hours on. Clone added at 0 hour. Fab of LB3.1 was used. Results are shown in **Fig. 18**.

Conclusion: Delayed addition of a peptide antagonist resulted in a gradual, time dependent abrogation of inhibitory activity, which could not be restored by increasing the concentration of competitor. In contrast, Fab fragments caused a near complete inhibition even when added 2 hours after the Th cells, and the

decreased inhibitory potency upon delayed addition could be compensated by incr ased Fab concentrations. Thus, Fab fragments seem to be more efficient in interfering with ongoing Th cell response than the currently available peptide competitors.

Example 22

Production of mAb 1-1C4

HLA-DR was immunoprecipitated from EBV-LCL Priess using mAb L243, and the HLA-DR α and ß chains were separated by SDS-PAGE. A 28k electrophoretic band containing DR-ß chain was cut from the gel and used to immunize a BALB/c mouse. Mouse immune B cells were subsequently immortalized by fusion with myeloma line PAI-0 [Stocker, W. et al., *Research Disclosure*, 217:155-157 (1982)] in order to obtain mAb secreting hybridomas. Culture supernatants of such hybridomas were screened for their capability to inhibit the activation of HA/DRB1*0401 Th cell clone KMHA25 (see Table 3) in the presence of antigen HA 307-319 and Priess as the APC. Hybridoma 1-1C4 was identified to be secreting a mAb having inhibitory capacity.

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Claims

1. A Fab fragment comprising an immunoglobulin Fab fragment and six complementarity-determining regions which are contained within said Fab fragment, wherein from one to six of said complementarity-determining regions are the complementarity-determining regions of a monoclonal antibody having the following properties:

1) the monoclonal antibody binds to the first domain of HLA-DR,

2) the monoclonal antibody is cytotoxic to antigen presenting cells which express HLA-DR,

3) the monoclonal antibody downregulates HLA-DR expression on the antigen presenting cells.

2. The Fab fragment of claim 1 wherein the monoclonal antibody is a murine monoclonal antibody.

3. The Fab fragment of claim 2 wherein the immunoglobulin is a human immunoglobulin.

4. The Fab fragment of claim 3 wherein the six complementarity-determining regions of said Fab fragment are the six complementarity-determining regions of said monoclonal antibody.

5. A fluid pharmaceutical composition comprising:

1) a pharmaceutically acceptable fluid carrier; and

2) a therapeutically effective amount of a Fab fragment as claimed in any one of claims 2 to 4.

6. The composition of claim 5 wherein the amount of the Fab fragment is from 0.5 to 5 mg/ml of the fluid composition.

7. The composition of claim 6 wherein the amount of the Fab fragment is from 1 to 2 mg/ml of the fluid composition.

8. A process for the preparation of a Fab fragment as defined in any one of claims 1 to 4 characterized in that a monoclonal antibody as defined in claims 1 or 2 is cleaved by pepsin and the Fab fragments are isolated by methods known in the state of the art.

9. A Fab fragment whenever prepared by the process of claim 8.

10. A Fab fragment according to any one of claims 1 to 4 as a therapeutically active agent, especially as an immunsuppressive agent.

11. Use of a Fab fragment according to any one of claims 1 to 4 for suppressing the immune response of a patient.

12. Use of a Fab fragment according to any one of claims 1 to 4 for the treatment of rheumatoid arthritis.

13. The invention as hereinbefore described.

14. A method for suppressing the immune response of a patient comprising administering a therapeutically effective amount of a Fab fragment comprising a Fab fragment as defined in claim 3 or 4.

***

Figure 1/1

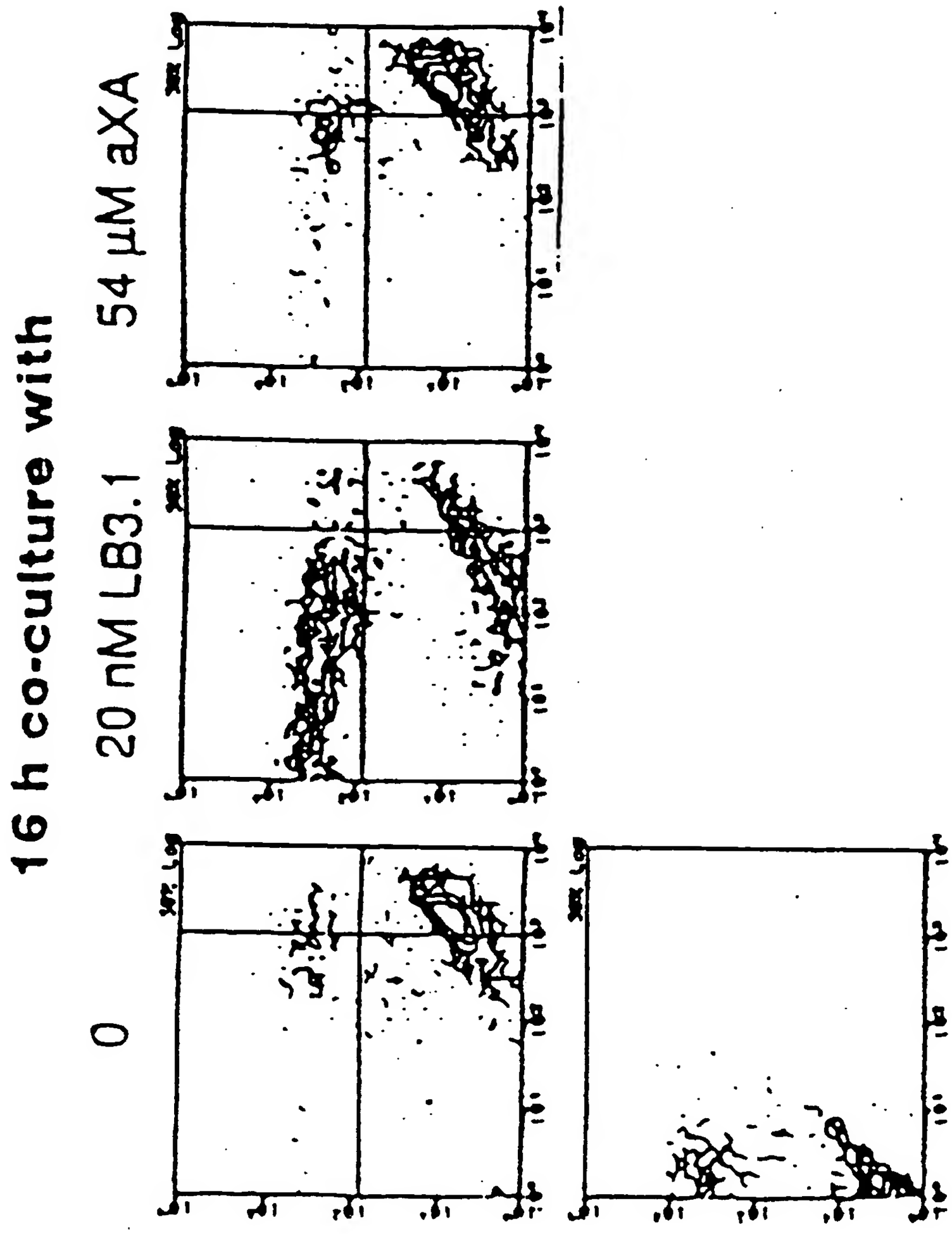

FITC staining
HLA-DR
Background
h co-culture with
20 nM LB3.1
54 µM aXA
16
0
2

Figure 1/2
16 h co-culture with

Figure 1/3

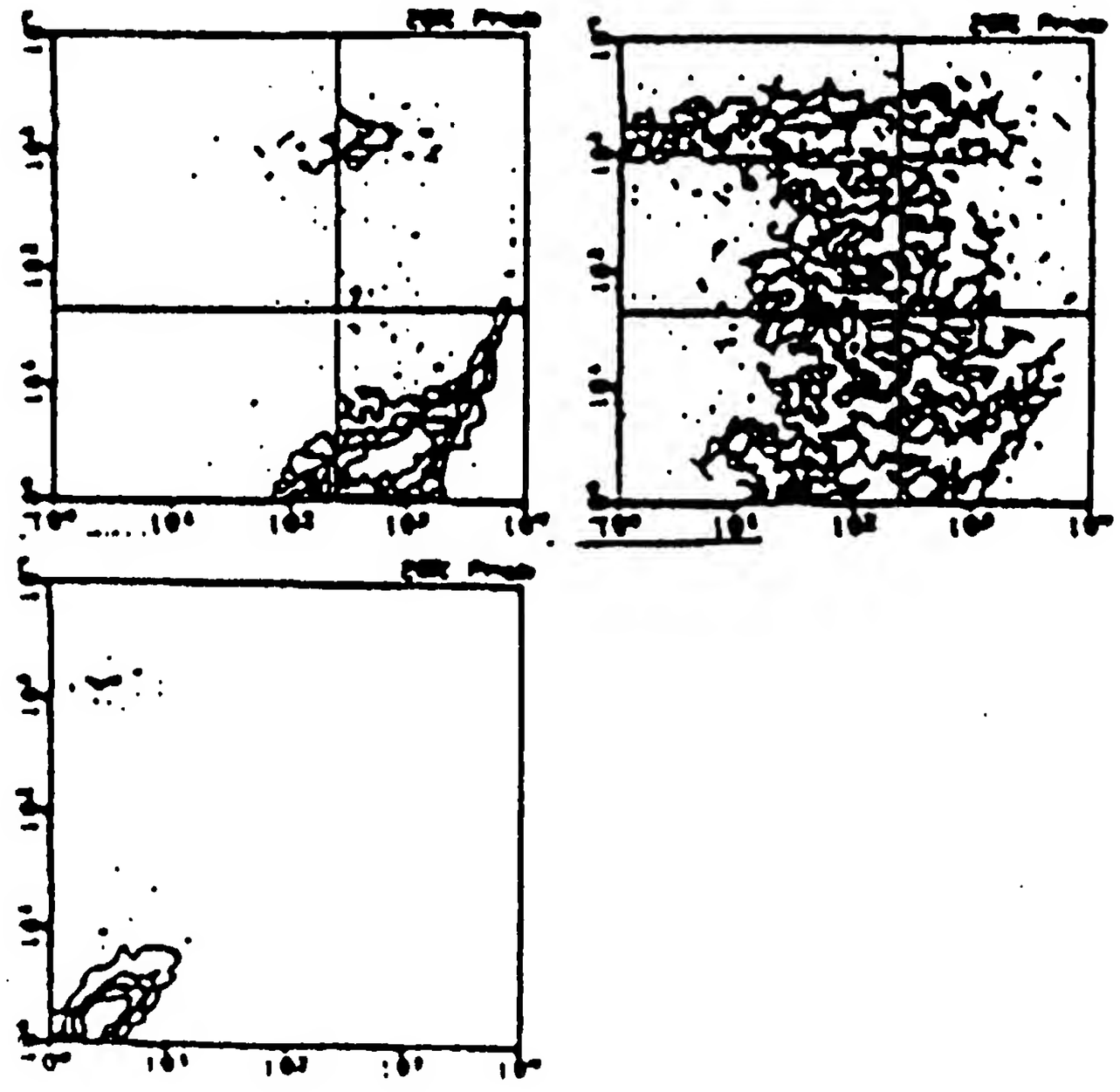
16 h co-culture with

FITC staining

C

0

20 nM 1-1C4



HLA-DR

Background

PCT/EP95/04648

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Figure 2

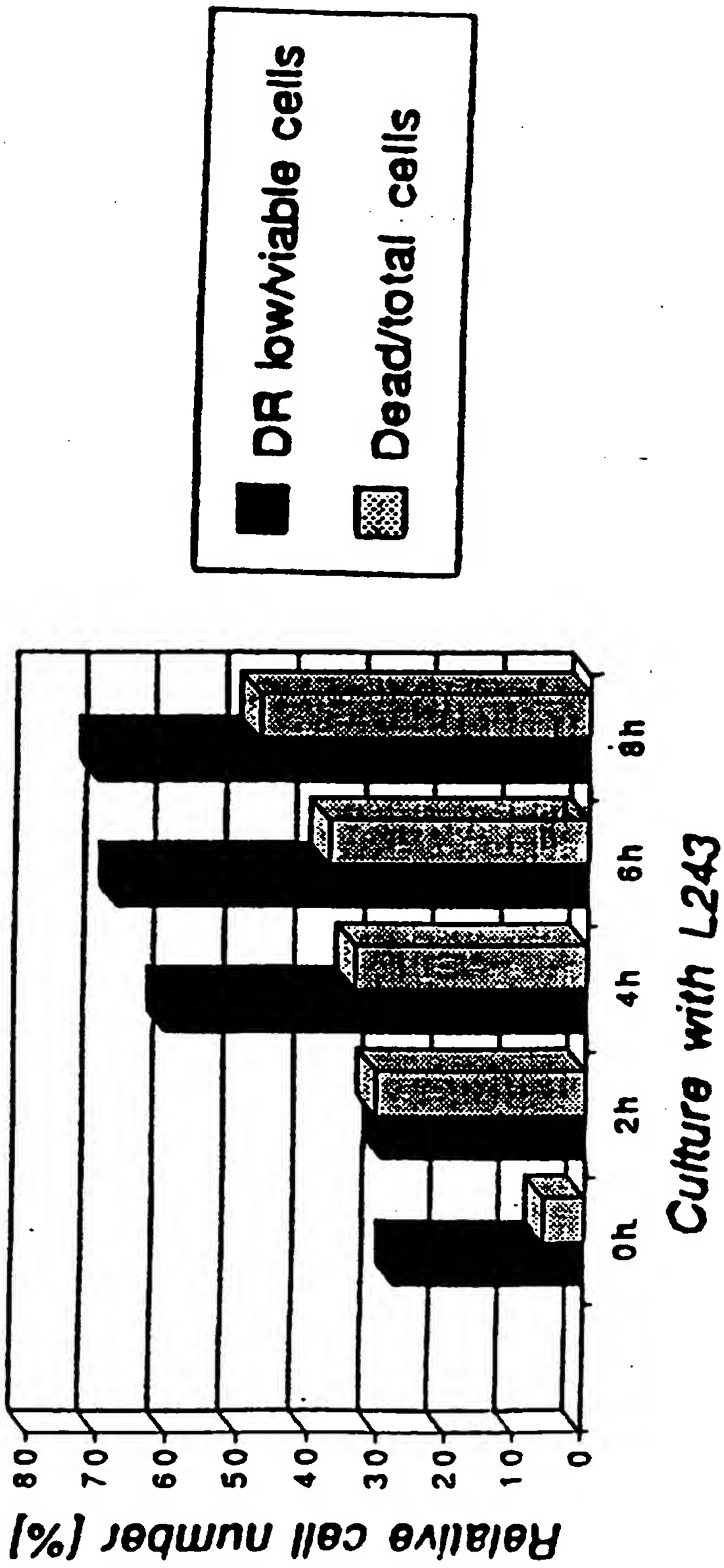

DR low/viable cells
Dead/total cells
Relative cell number [%]
0
10
20
30
40
50
60
70
80
0h
2h
4h
6h
8h
Culture with L243

Figure 3

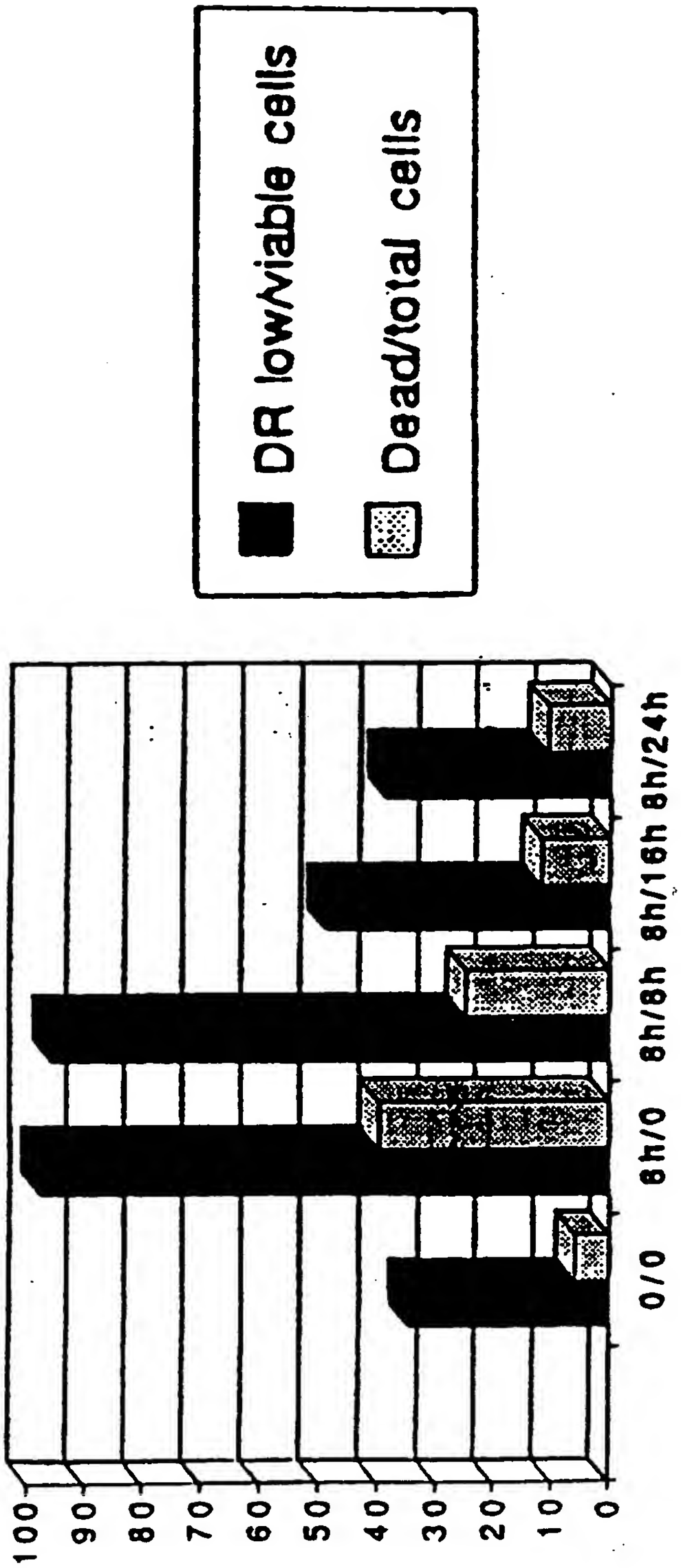

DR low/viable cells
Dead/total cells
0/0
8h/0
8h/8h
8h/16h
8h/24h
Time with/without L243
100
90
80
70
60
50
40
30
20
10
0
Relative cell number [%]

Figure 4

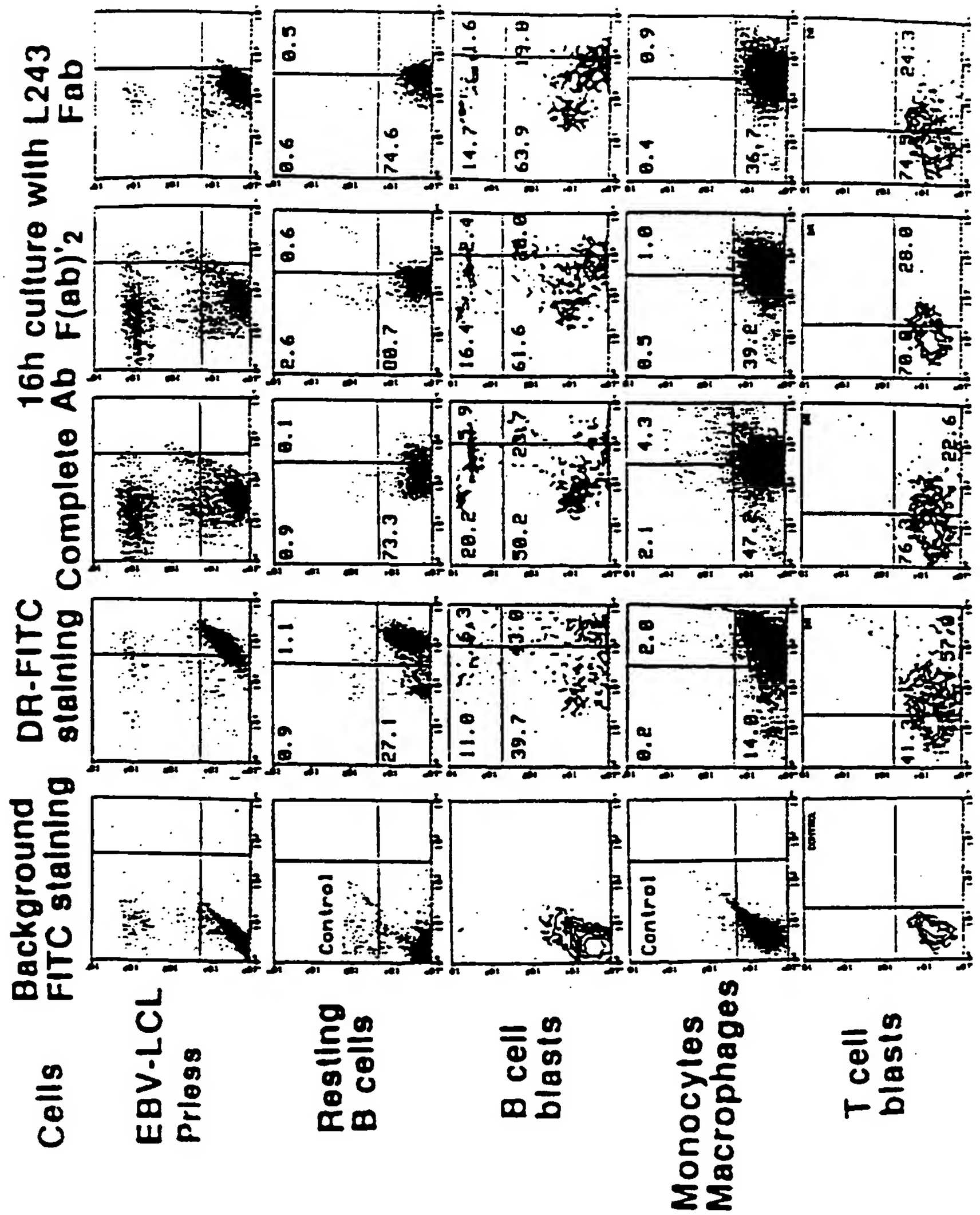
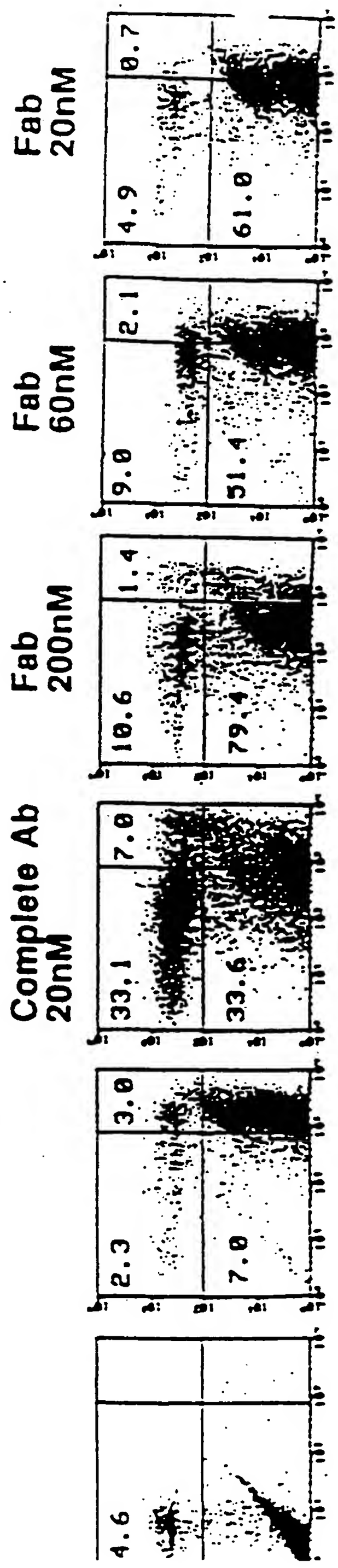

Background DR-FITC staining
16h culture with L243 Fab
16h culture with L243 F(ab)'2
16h culture with Complete Ab
Cells
EBV-LCL
Prioss
Resting B cells
B cell blasts
Monocyles Macrophages
T cell blasts
Control
Control

Figure 5

Priess



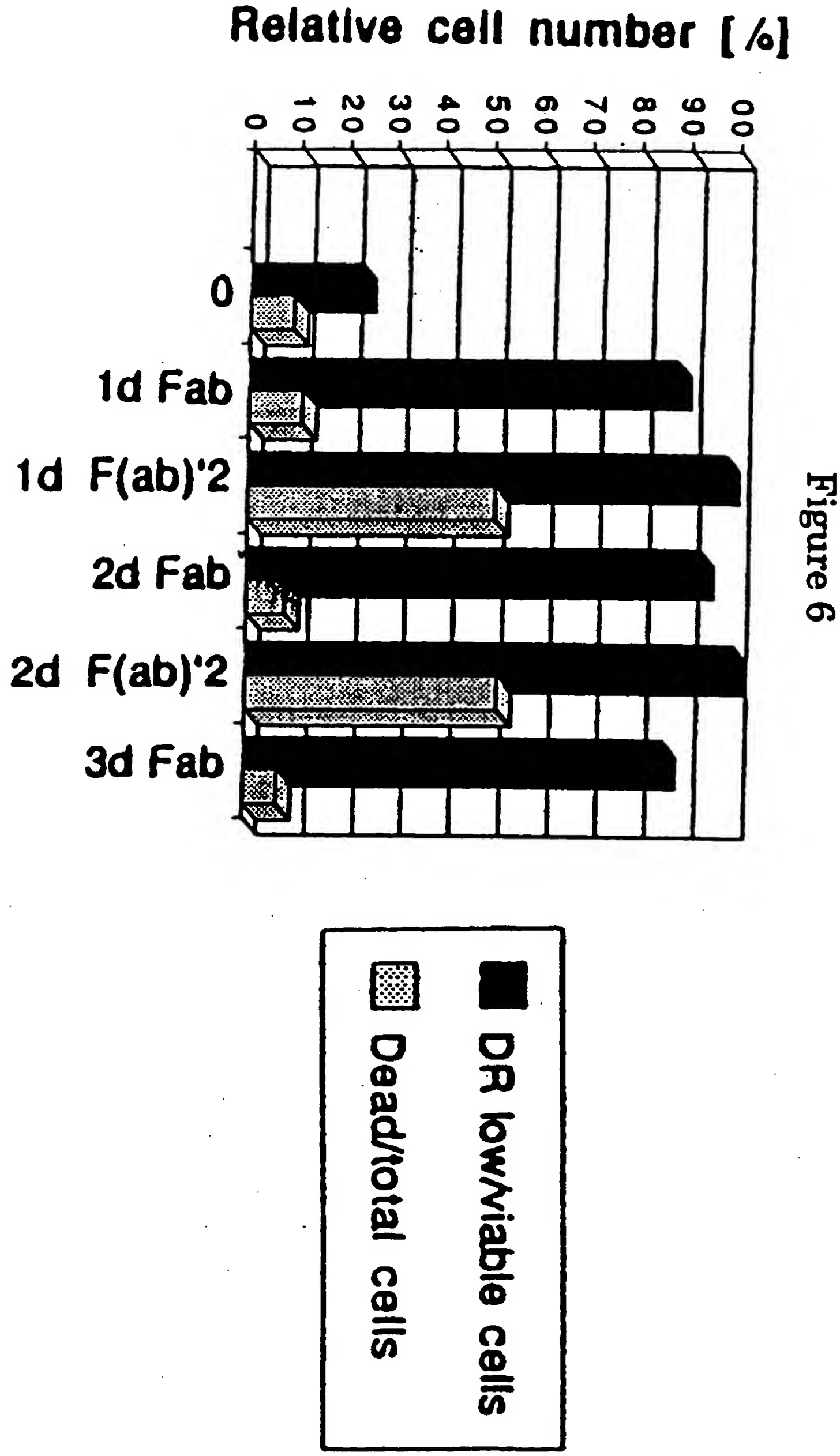

Relative cell number [%]
0
10
20
30
40
50
60
70
80
90
00
0
1d Fab
1d F(ab)'2
2d Fab
2d F(ab)'2
3d Fab
DR low/viable cells
Dead/total cells

Figure 6

Figure 7

EBV-LCL PRIESS

16 h co-culture with L243

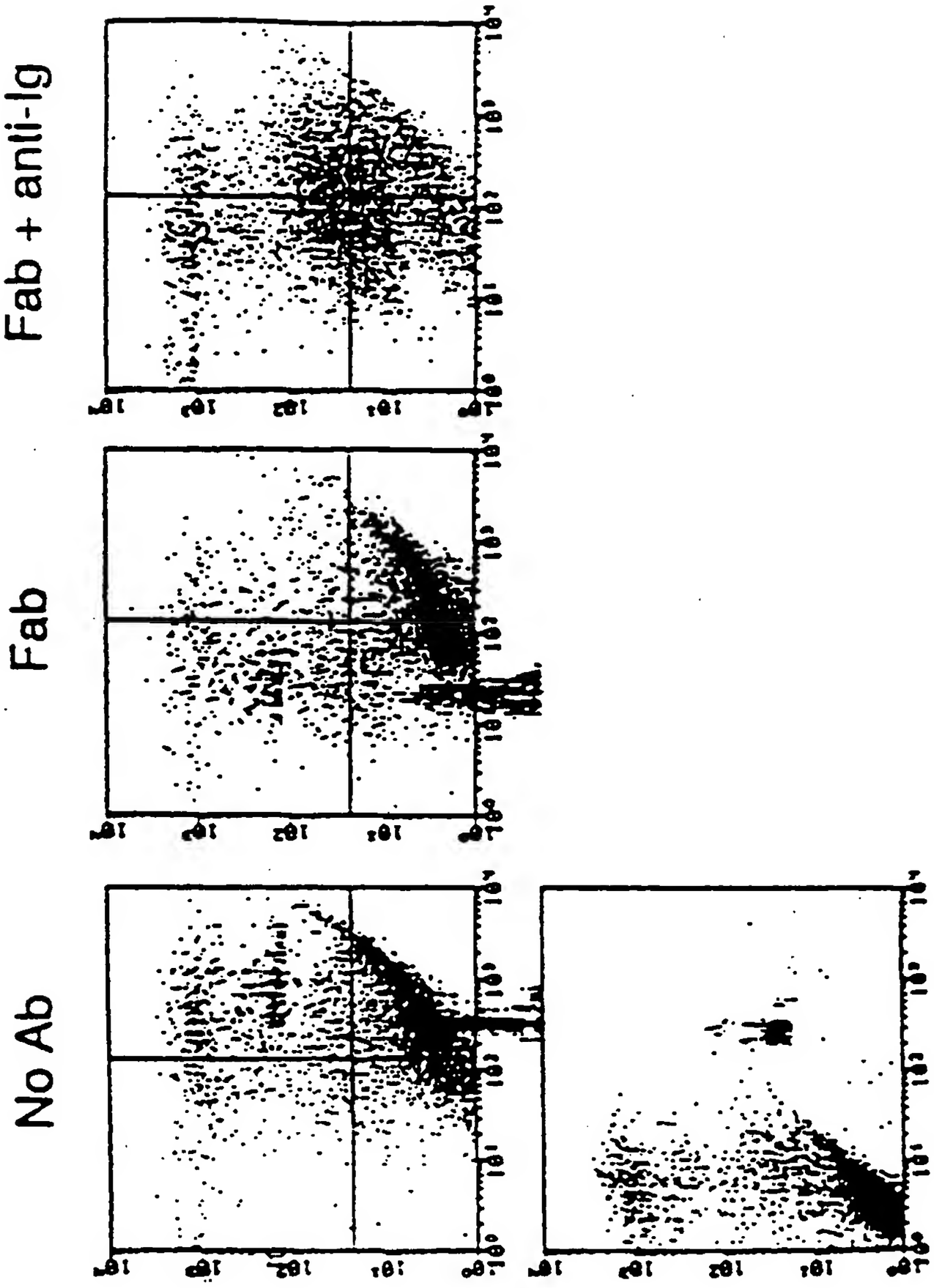


Figure 8

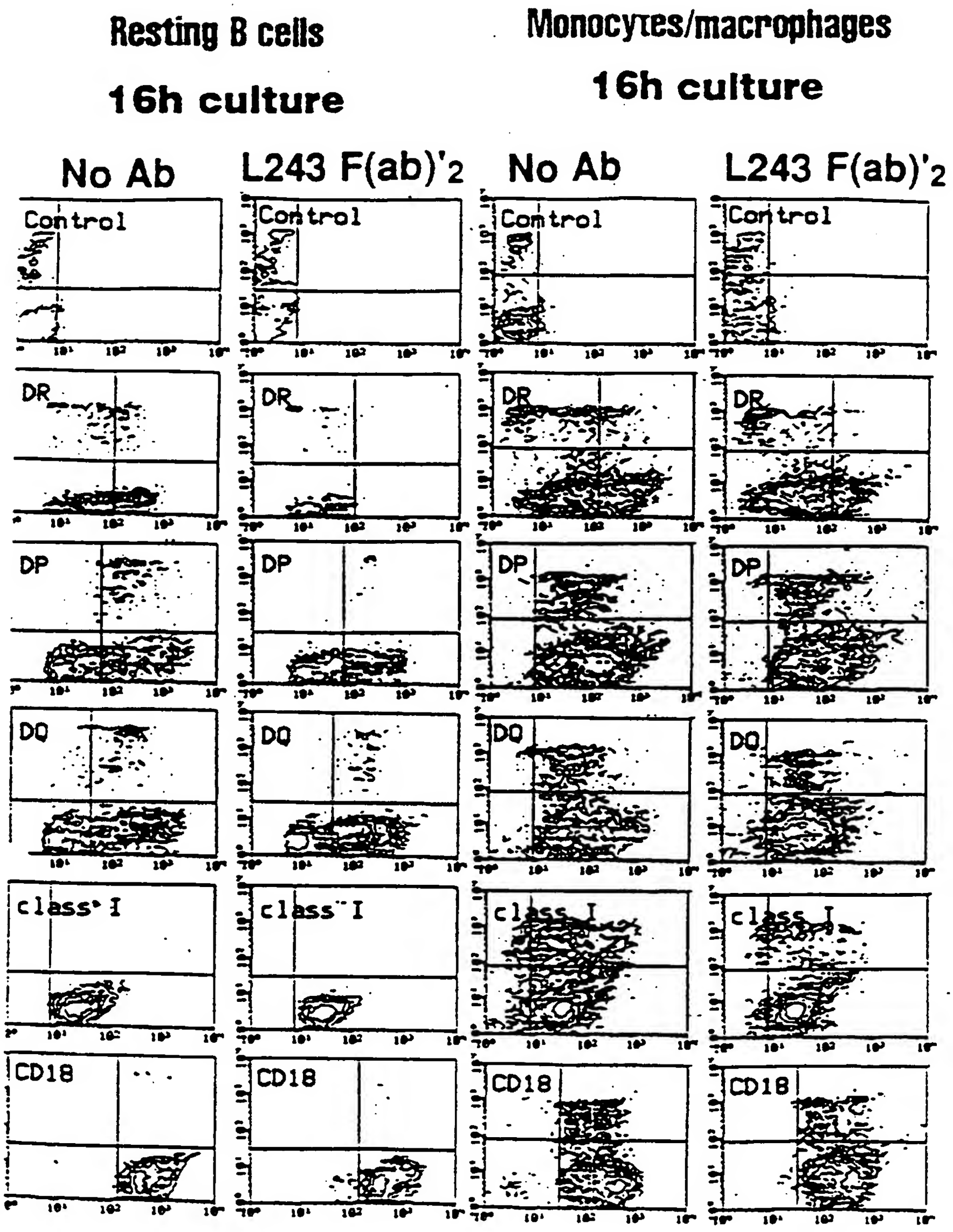

Resting B cells
16h culture
Monocytes/macrophages
16h culture
No Ab
L243 F(ab)'2
No Ab
L243 F(ab)'2
Control
DR
DP
DQ
class I
CD18

Figure 9

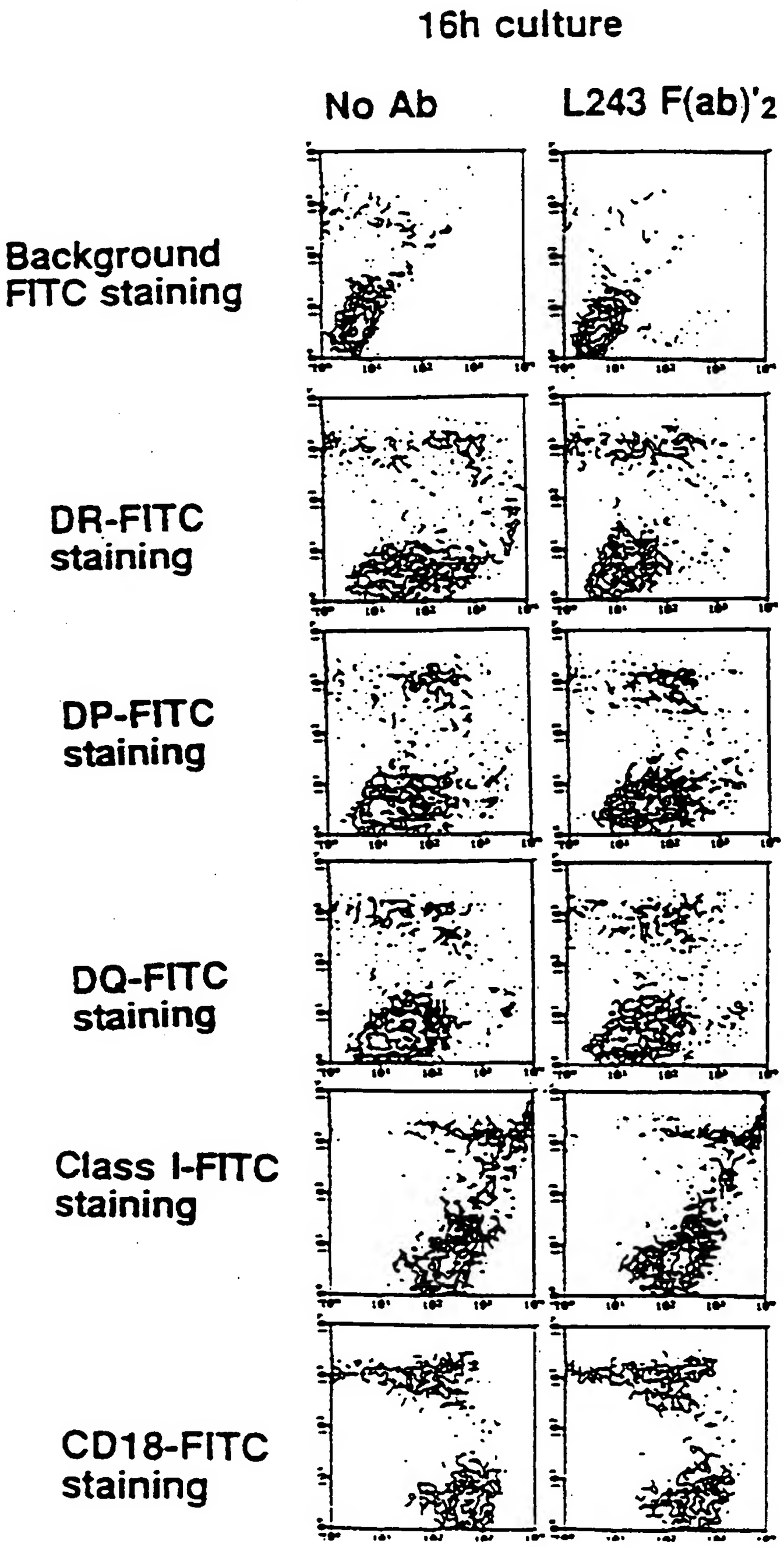

16h culture
No Ab
L243 F(ab)'2
Background FITC staining
DR-FITC staining
DP-FITC staining
DQ-FITC staining
Class I-FITC staining
CD18-FITC staining

Figure 10

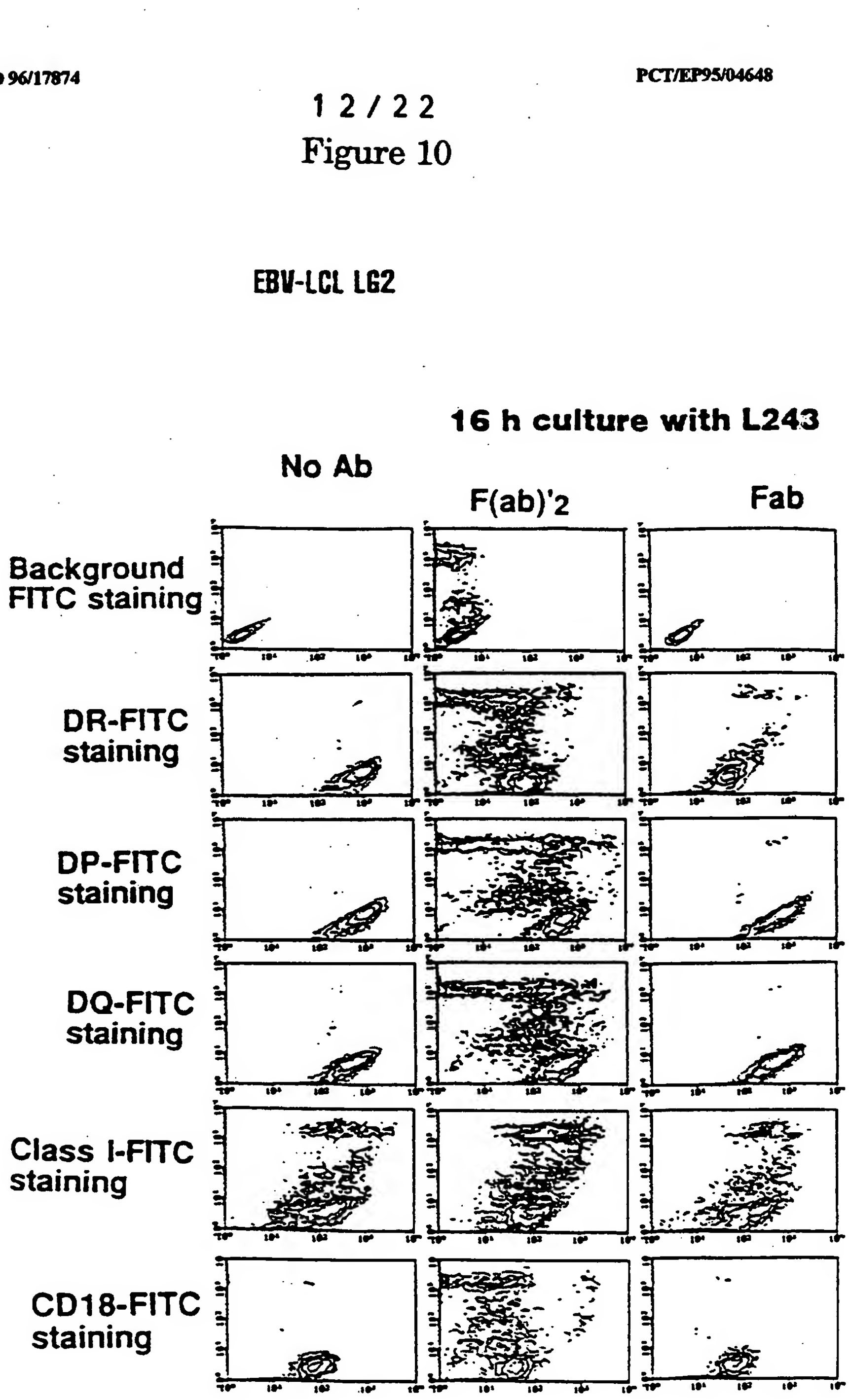

EBV-LCL LG2
16 h culture with L243
No Ab
F(ab)'2
Fab
Background FITC staining
DR-FITC staining
DP-FITC staining
DQ-FITC staining
Class I-FITC staining
CD18-FITC staining

Figure 11

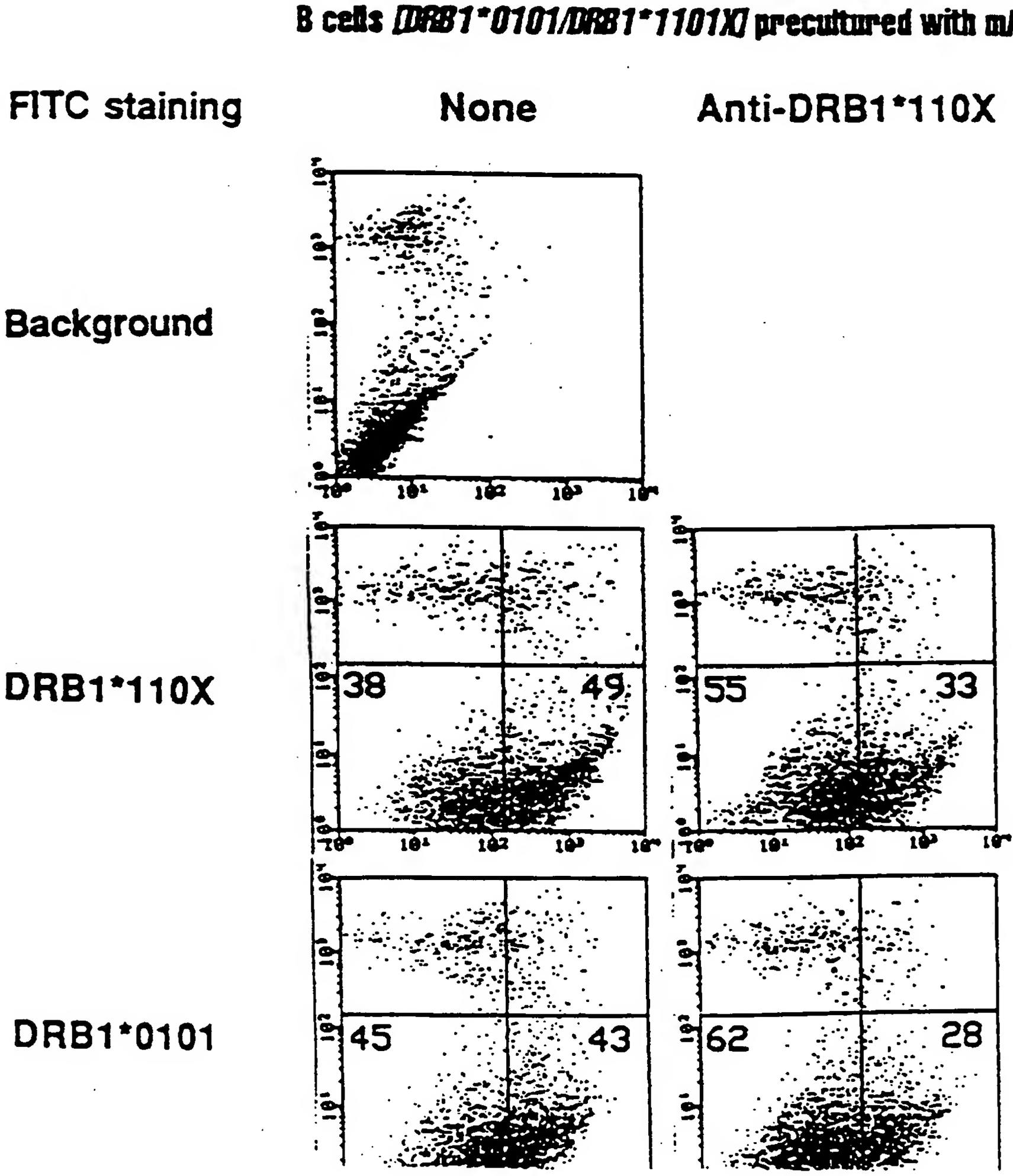
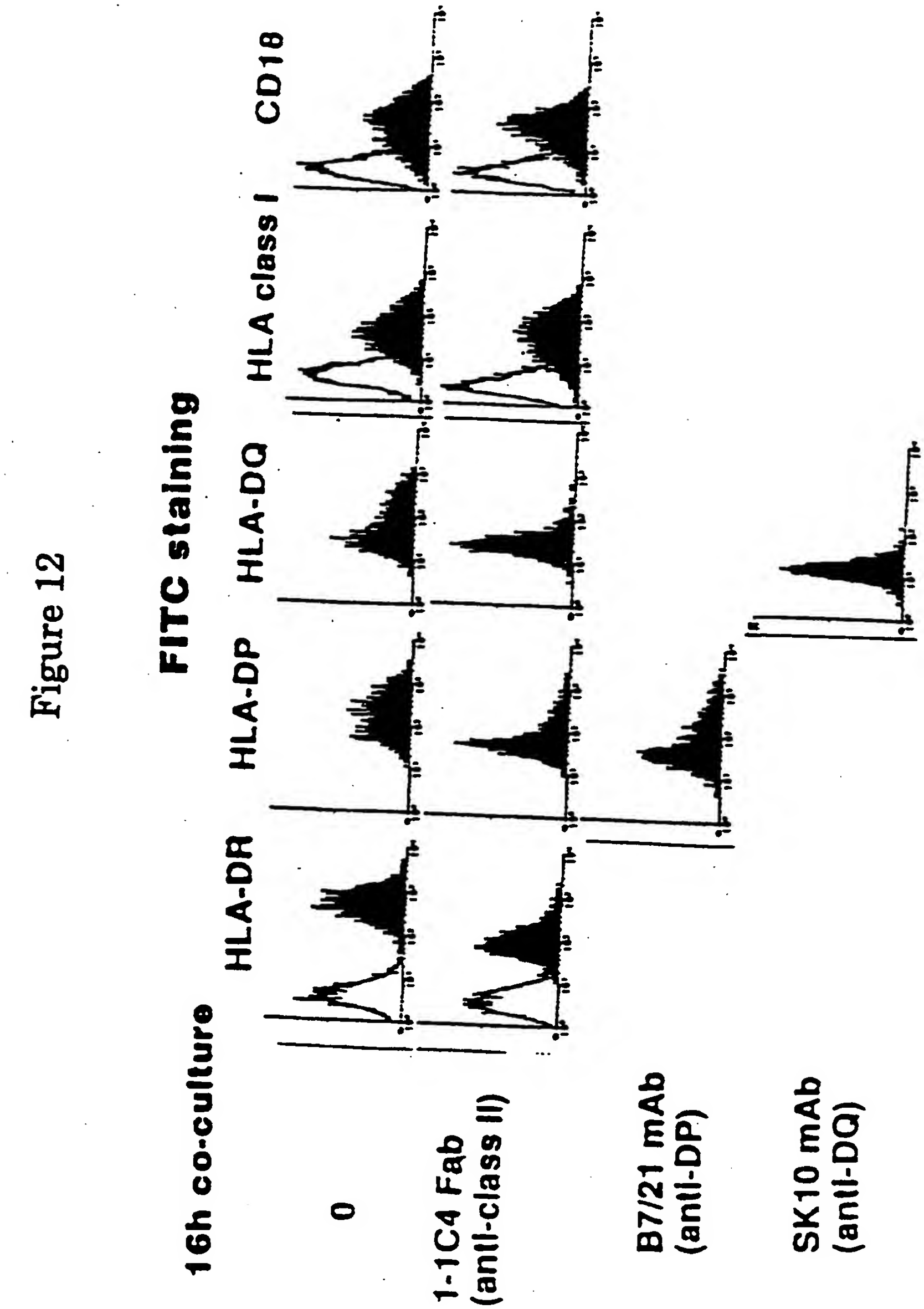

B cells DRB1*0101/DRB1*1101X precultured with mAb
FITC staining
None
Anti-DRB1*110X
Background
DRB1*110X
38
49
55
33
DRB1*0101
45
43
62
28

Figure 12


16h co-culture
FITC staining
HLA-DR
HLA-DP
HLA-DQ
HLA class I
CD18
0
1-1C4 Fab
(anti-class II)
B7/21 mAb
(anti-DP)
SK10 mAb
(anti-DQ)

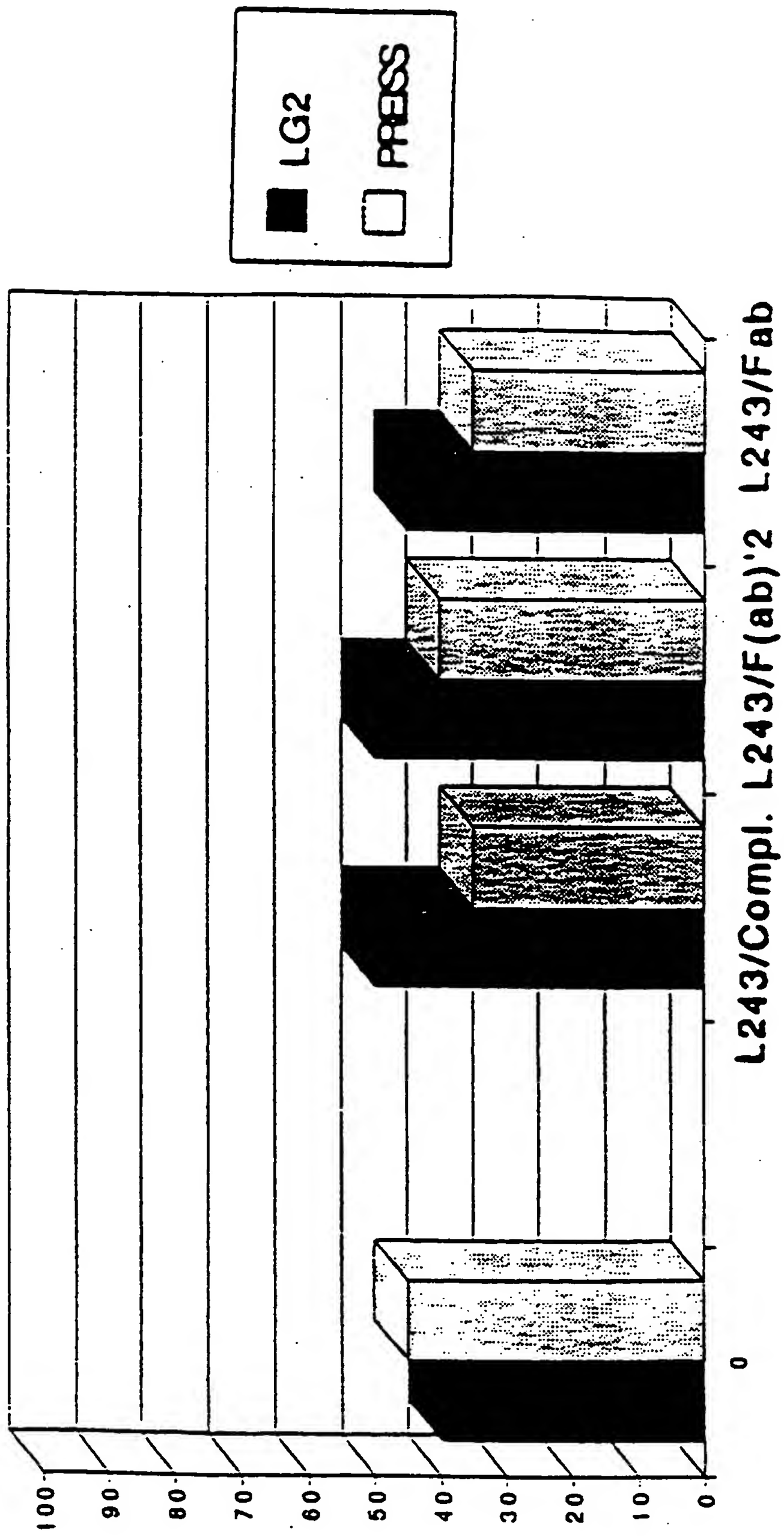

LG2
PRESS
L243/Fab
L243/Compl. L243/F(ab)'2
0
10
20
30
40
50
60
70
80
90
100
TNF-alpha [pg/ml]

Figure 13

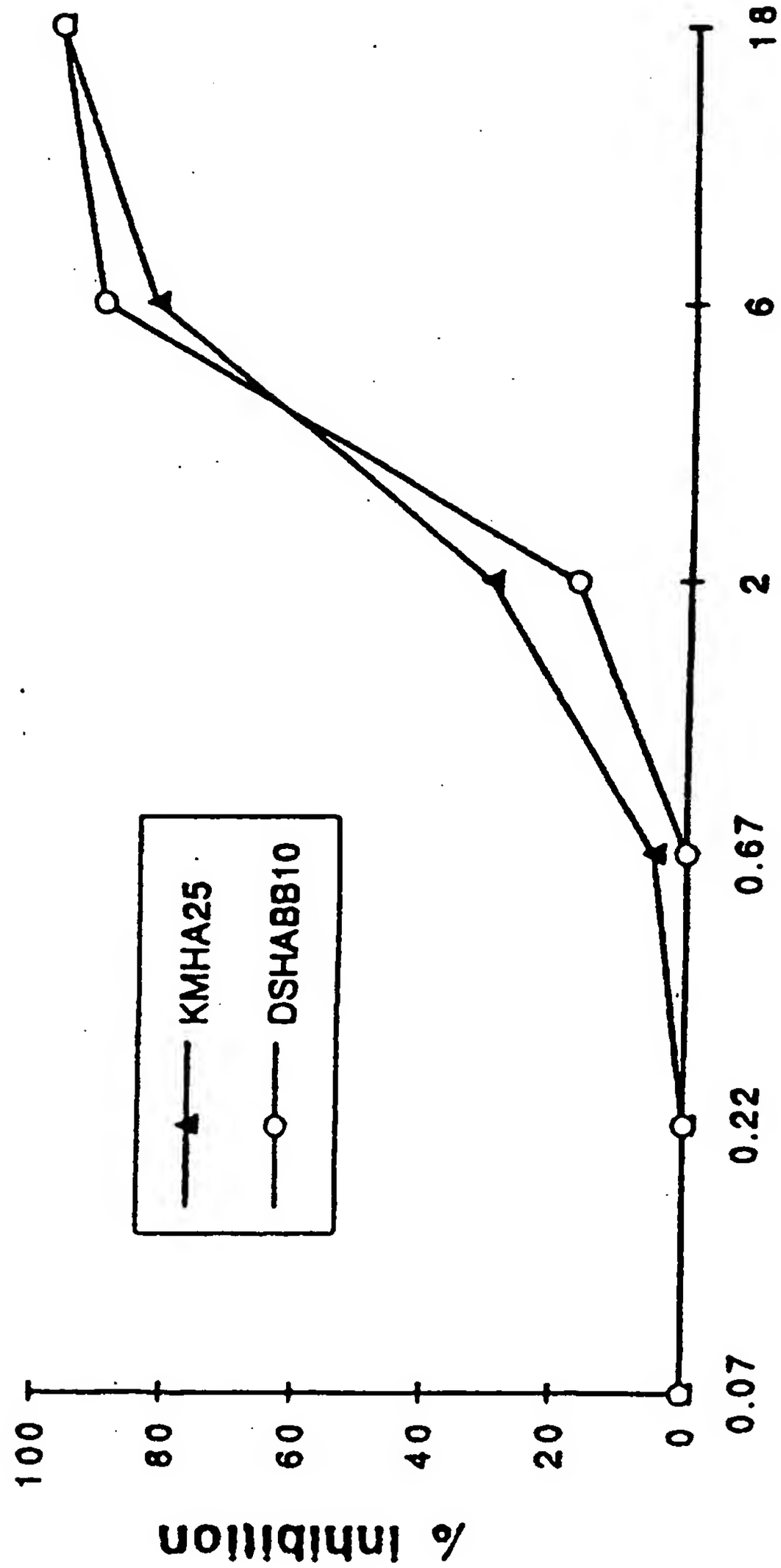

KMHA25
DSHA8B10
100
80
60
40
20
0
% inhibition
0.07
0.22
0.67
2
6
18
nM LB3.1

Figure 14/1

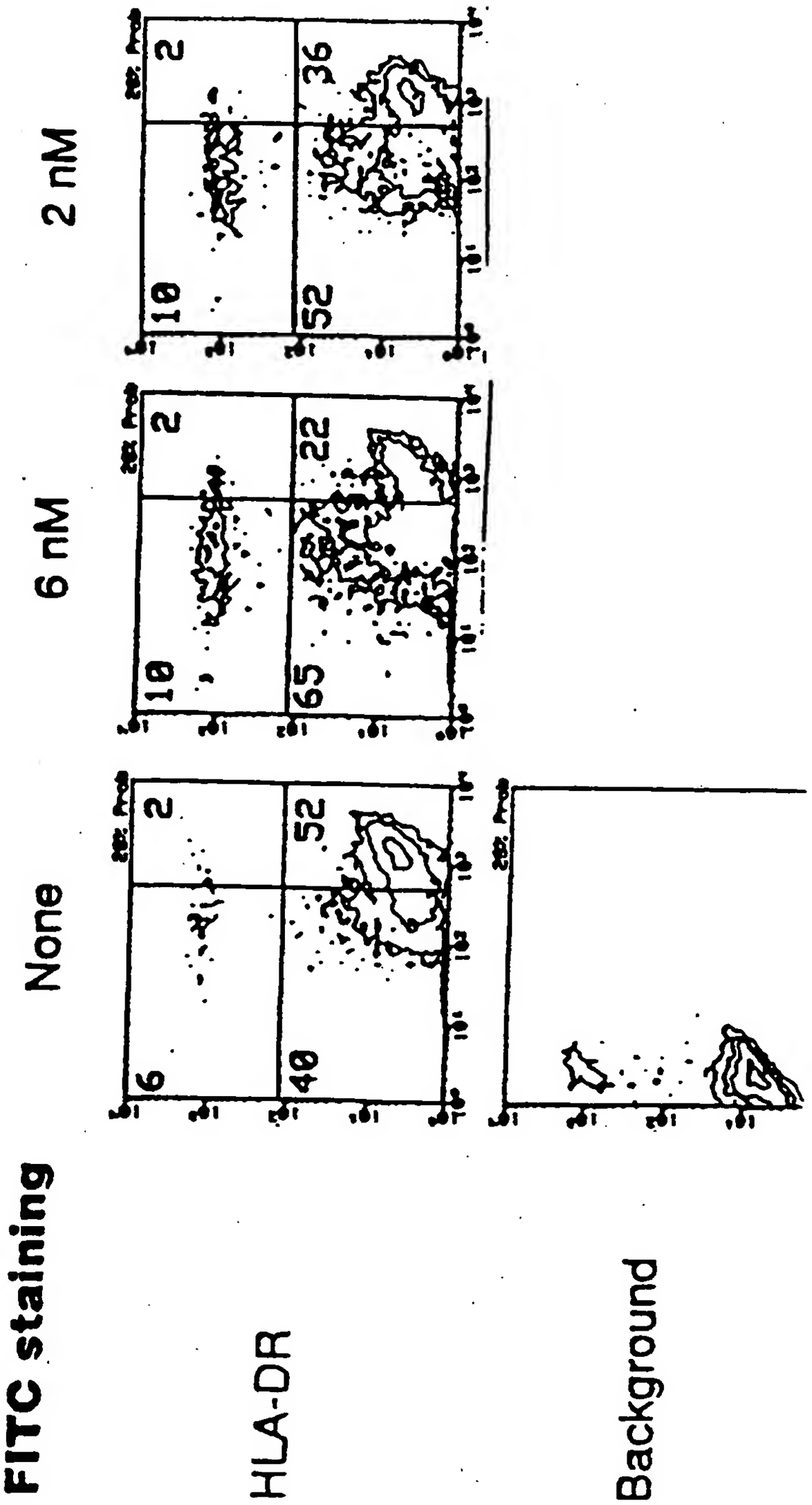
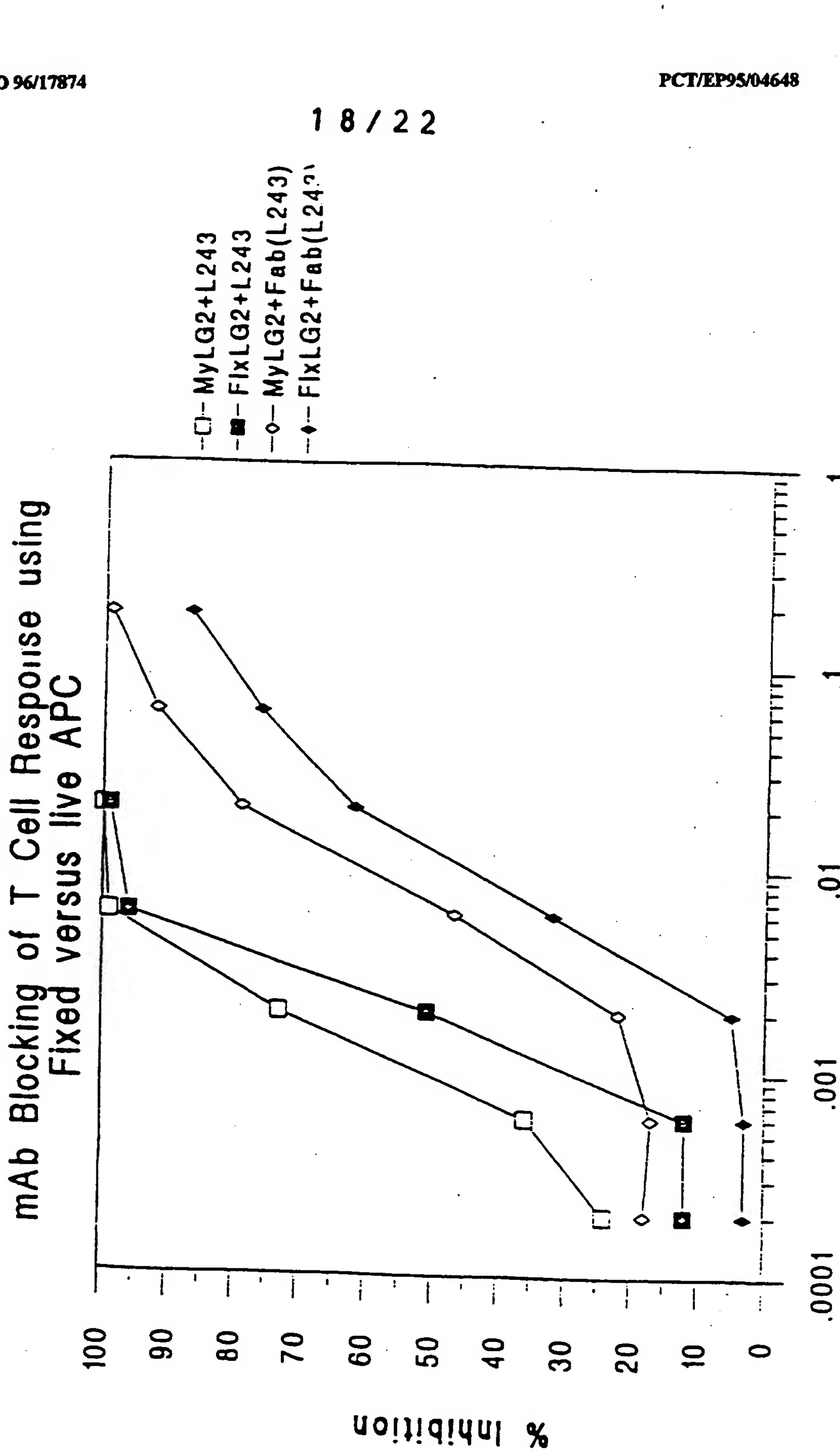

FITC staining
HLA-DR
Background
None
6 nM
2 nM
16 h co-culture with LB3.1
2
6
40
52
2
10
65
22
2
10
52
36

Figure 14/2

Figure 15


mAb Blocking of T Cell Response using Fixed versus live APC
-□- MyLG2+L243
-■- FIxLG2+L243
-○- MyLG2+Fab(L243)
-◆- FIxLG2+Fab(L24?)
uM mAb
.0001
.001
.01
.1
1
% Inhibition
100
90
80
70
60
50
40
30
20
10
0

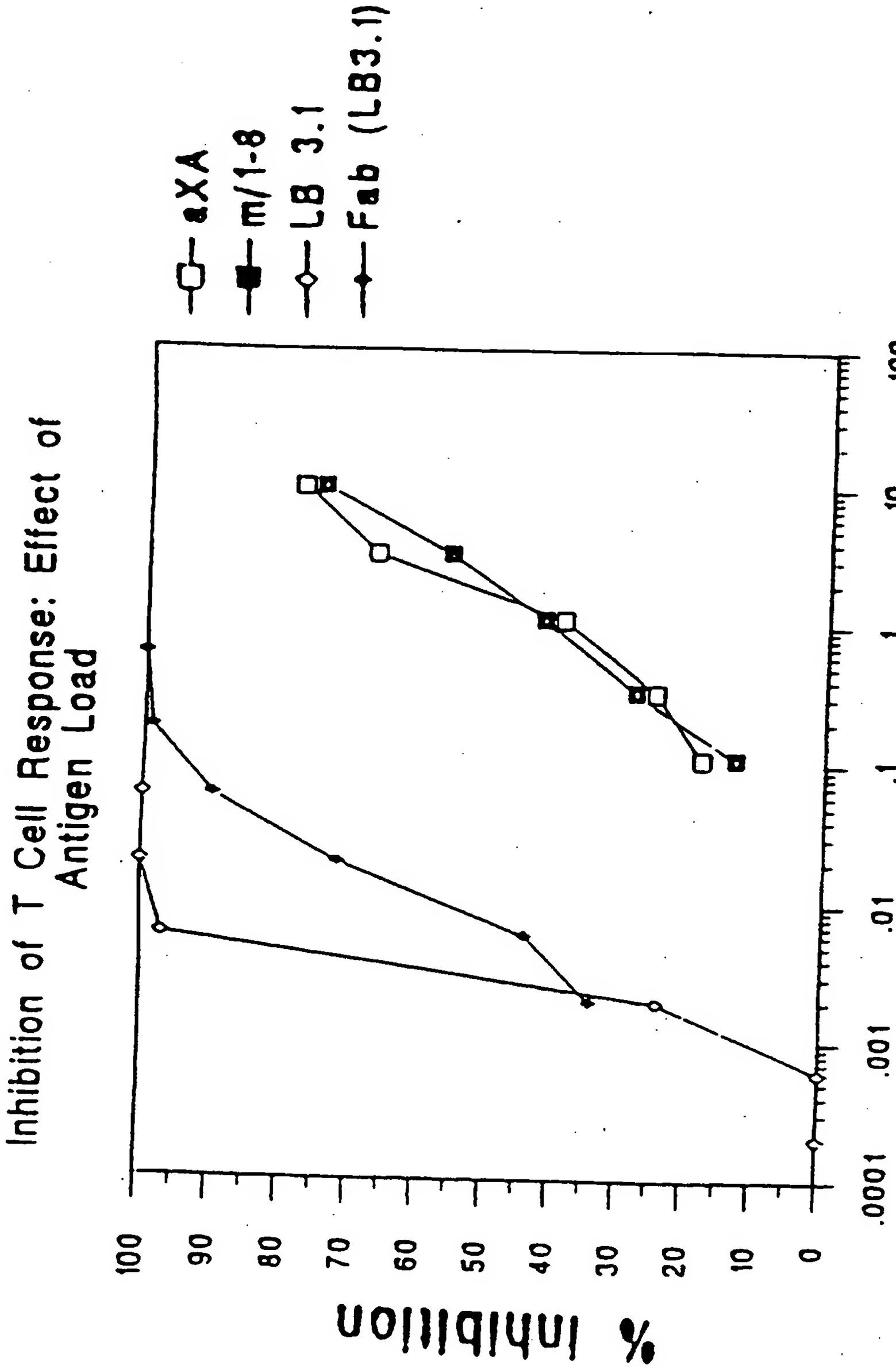

αXA
m/1-8
LB 3.1
Fab (LB3.1)
Inhibition of T Cell Response: Effect of Antigen Load
% Inhibition
100
90
80
70
60
50
40
30
20
10
0
100
10
1
.1
.01
.001
.0001
uM blocker

Figure 16/1

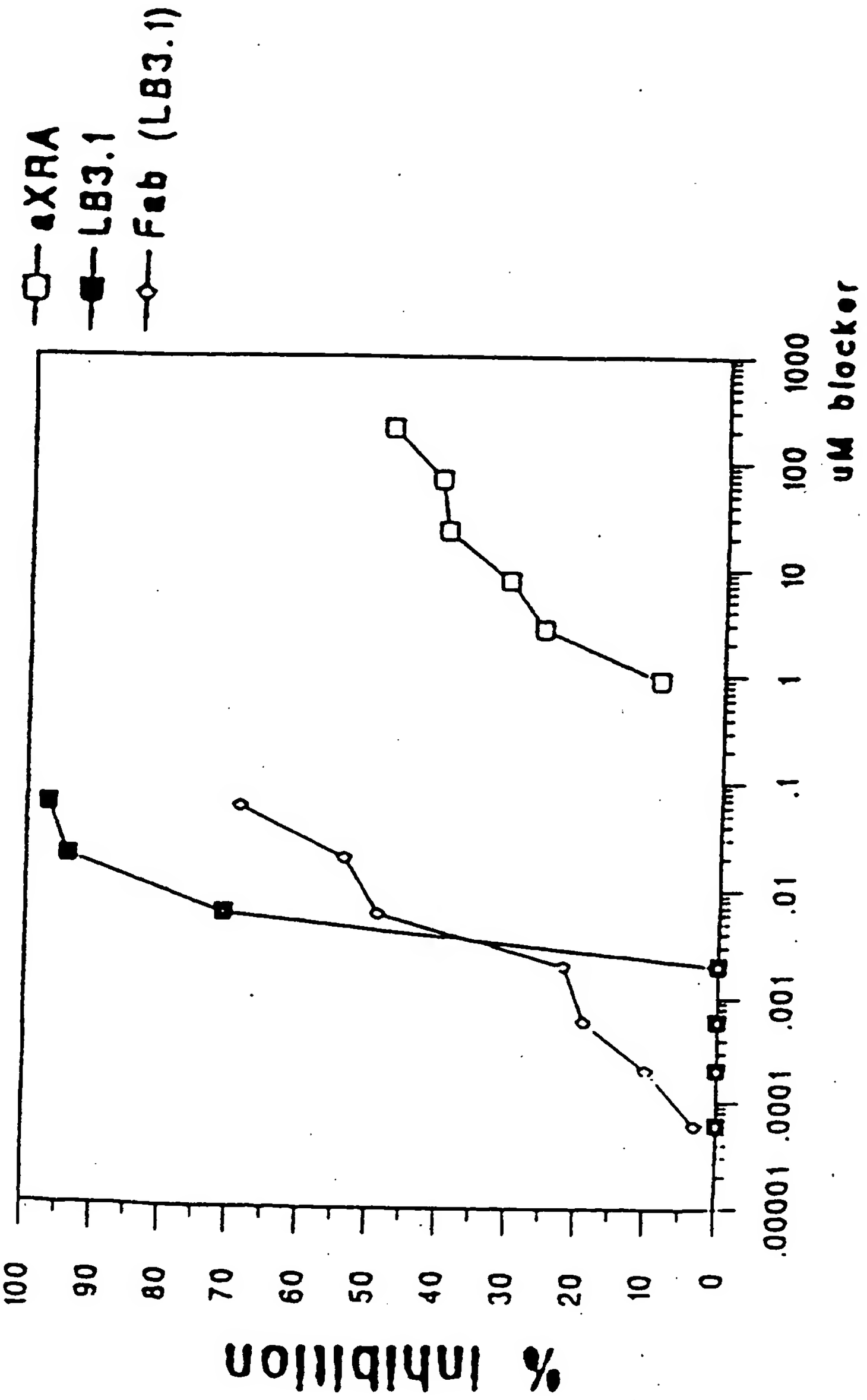

αXRA
LB3.1
Fab (LB3.1)
1000
100
10
1
.1
.01
.001
.0001
.00001
uM Mn
100
90
80
70
60
50
40
30
20
10
0
% inhibition

Figure 16/2

Figure 17

Comparison of Peptide Antagonist and Fab(L243) in Inhibition of T Cell Response

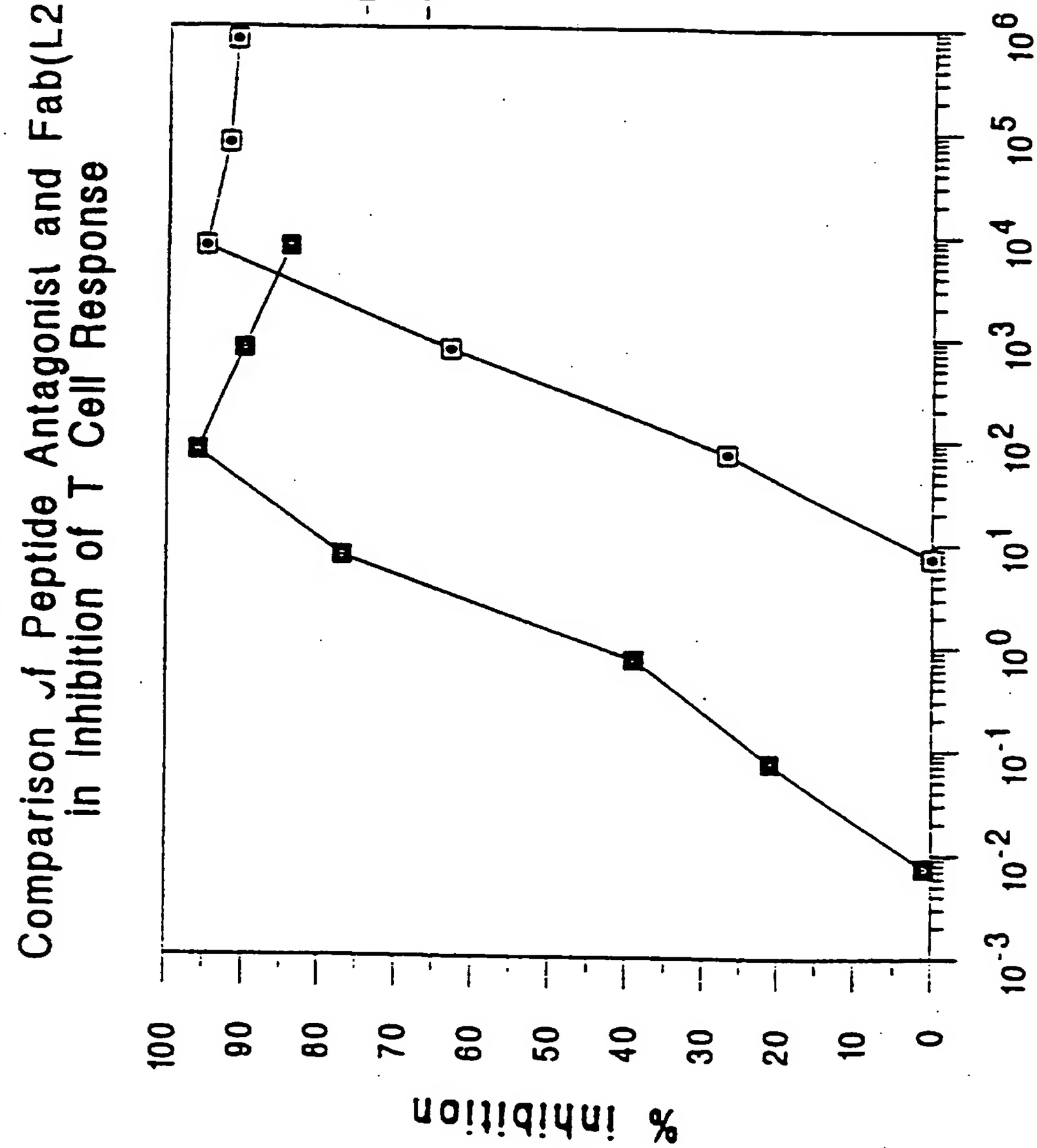
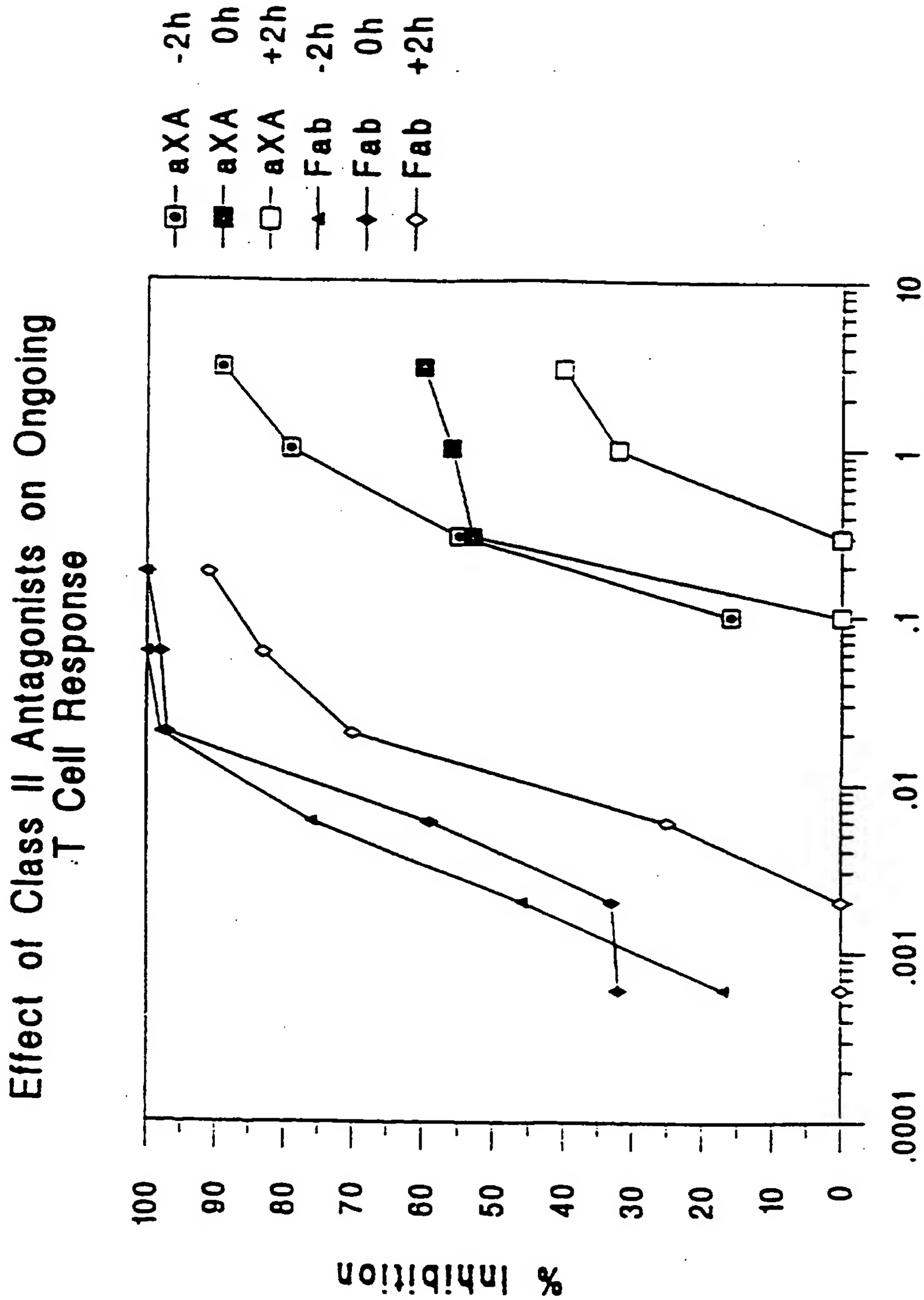


Figure 18

Effect of Class II Antagonists on Ongoing T Cell Response



INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/EP 95/04648

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/28 A61K39/395 C12P21/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARTHRITIS AND RHEUMATISM, vol. 26, no. 4, April 1983 NEW YORK, NY, USA, pages 486-493, R. SEARLES ET AL. 'Ia-specific antilymphocyte antibodies in rheumatoid arthritis.' see abstract ---	1-14
A	EP,A,0 068 790 (THE BOARD OF TRUSTEES OF LELAND STANFORD JUNIOR UNIVERSITY) 5 January 1983 see page 8, line 10 - page 9, line 16 see claims --- -/--	1-14

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 February 1996	26.03.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

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INTERNATIONAL SEARCH REPORT

Interr al Application No
PCT/EP 95/04648

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TRANSPLANTATION PROCEEDINGS, vol. 15, no. 1, March 1983 NEW YORK, NY, USA, pages 649-650, XP 000564476 R. BILLING ET AL. 'Prolongation of skin allograft survival in monkeys treated with anti-Ia and anti-blast/monocyte monoclonal antibodies.' cited in the application see the whole document ---	1-14
A	WO,A,93 02108 (IDEC PHARMACEUTICALS CORPORATION) 4 February 1993 see page 10, line 24 - page 11, line 7 see page 38, line 31 - page 39, line 21 see claims ---	1-14
A	THE JOURNAL OF IMMUNOLOGY, vol. 135, no. 4, October 1985 BALTIMORE, MD, USA, pages 2393-2399, R. HOHLFELD ET AL. 'Genetic restriction of autoreactive acetylcholine receptor-specific T lymphocytes in myastenia gravis.' see the whole document ---	1-14
A	WO,A,91 10722 (CENTOCOR INC.) 25 July 1991 see the whole document ---	1-14
T	EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 25, no. 12, December 1995 WEINHEIM, GERMANY, pages 3349-3355, XP 000564479 D. VIDOVIC ET AL. 'Down-regulation of class II major histocompatibility complex molecules on antigen-presenting cells by antibody fragments.' see the whole document -----	1-14

1

INTERNATIONAL SEARCH REPORT

! national application No.
PCT/EP 95/04648

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 11,12,14
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 11, 12 and 14 are directed to a method of treatment of the human/animal body, the search has beencarried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter nal Application No
PCT/EP 95/04648

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-68790	05-01-83	JP-A-	58099422	13-06-83
WO-A-9302108	04-02-93	AP-A-	307	31-01-94
		AU-B-	2425592	23-02-93
		BG-A-	98411	28-02-95
		BR-A-	9206313	11-04-95
		CA-A-	2114015	04-02-93
		CZ-A-	9400149	13-07-94
		EP-A-	0605442	13-07-94
		FI-A-	940336	10-03-94
		HU-A-	70272	28-09-95
		JP-T-	6509708	02-11-94
		NO-A-	940219	25-03-94
		NZ-A-	243706	26-08-94
		OA-A-	9879	15-09-94
		PT-A-	100735	29-10-93
		SK-A-	8894	07-09-94
WO-A-9110722	25-07-91	CA-A-	2070182	28-06-91
		EP-A-	0511308	04-11-92